ANALGESIC AND IMMUNOMODULATORY EFFECTS OF CODEINE AND CODEINE 6-GLUCURONIDE

By

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The interactions between opioid analgesics and the human immune system can have important clinical consequences. A better understanding of these interactions is needed due to the widespread use and abuse of opiates. In recent years, an increased knowledge and awareness in this area has generated a considerable surge in research. Narcotics are predominantly used to alleviate pain and discomfort in patients with trauma or undergoing major surgery. However, they are also known to cause impairment of the immune system. Subsequently, this could lead to patients becoming predisposed to infectious diseases as a result of the immunosuppressive effects of narcotics.

An HPLC system was successfully developed for the analysis of codeine and its metabolites in various biological samples, that is, plasma, urine and brain tissue. Codeine 6-glucuronide and an intermediate compound were synthesized using a modification of the Koenigs-Knorr reaction. The synthetic procedure was

efficient and reproducible. Analgesia studies with the tail flick method showed that codeine 6-glucuronide and the intermediate exhibited a higher analgesic activity compared to codeine when administered intracerebroventricularly. However, both compounds were not as active as codeine when administered by subcutaneous and intravenous routes. Immunomodulatory studies showed that the glucuronide metabolites of codeine and morphine were less immunosuppressive compared to their parent compounds, especially at physiologically relevant concentrations. Receptor binding profiles of codeine 6-glucuronide and the intermediate were similar to codeine, indicating that they possessed activity towards the μ-opioid receptors.

The overall goal of the project was to correlate the analgesic and immunomodulatory effects of codeine and codeine 6-glucuronide. This would result in a better understanding of the significance of high levels of codeine 6-glucuronide present in the plasma and urine in man after codeine administration. Further, this may lead to the development of glucuronide analogs for the management and treatment of pain in immunocompromised patients.

CHAPTER 1
BACKGROUND AND SIGNIFICANCE

Pain is an unpleasant sensation that can disturb the comfort, thought, sleep, and normal daily activity of a person. Pain signals are considered to be part of a protective mechanism designed to indicate the presence of a potentially dangerous condition. Thus, it is considered to be symptomatic of an underlying condition that requires attention and treatment. Pain is the net effect of complex interactions of ascending and descending neurosystems which include biochemical, physiological, psychological, and neurocortical processes. Also, since pain is a very subjective experience, only the patient can describe its intensity. This subjectivity makes it difficult to assess the activity of analgesics in humans.

Analgesics are defined as drugs that can relieve pain without causing loss of consciousness. The most potent analgesics are referred to as narcotics and act directly on the central nervous system. Narcotics as a group include the opioids, which are considered to be the most effective analgesics available. The opioid family, whose name derives from opium, includes agents such as morphine, codeine, meperidine and methadone. While opioid is a general term for any drug, natural or synthetic, that has actions similar to morphine, the term opiate is more specific and applies only to compounds present in opium such as morphine and codeine. Apart from acting as analgesics, opioids produce a variety of pharmacological actions on various tissues in the body.

1.1 Opioids and Pain

Opioids represent the main class of drugs in the clinical management of mild to moderate pain in various cases of medical illness, and relieve pain primarily through direct actions on receptors in the central nervous system. Opioid analgesics include natural alkaloids from opium (morphine, codeine), synthetic surrogates (methadone, meperidine) and endogenous peptides (enkephalins, β-endorphins).

Opioids act at receptor sites both within and outside the central nervous system. Binding studies with various drugs and ligands in the brain and other tissues suggest the presence of a multitude of opioid receptors. The three important receptor types are designated as mu (μ), kappa (κ) and delta (δ). The effects mediated by the μ receptors include supraspinal analgesia, respiratory depression and euphoria. The κ receptors mediate analgesia at the level of the spinal cord, along with sedation and miosis. The δ receptors are also thought to be involved in analgesia, both at the spinal and supraspinal sites. However, their role in this regard remains controversial (Jaffe and Martin, 1985).

The body produces three families of peptides that are capable of interacting with opioid receptors--enkephalins, β-endorphins and dynorphins. These endogenous opioids have a high affinity for the μ, κ and δ receptors, respectively. They are present throughout the body and serve as hormones and neurotransmitters. It is thought that morphine and other opioid analgesics mimic the actions of these endogenous ligands by binding to the opioid receptors. These interactions are presumed to give rise to the observed pharmacological effects.

1.1.1 Pain Transmission

Pain generally begins with a noxious stimulus that injures or destroys tissues. Endogenous chemical substances such as histamine, bradykinins, prostaglandins, and others are then released from the damaged tissues and nerve terminals. The released chemicals bind to "pain receptors" or nociceptors present along the afferent nerve fibers, depolarizing the nerve membranes and initiating an action potential. This causes the generation of a pain impulse which is then transmitted via the afferent fibers to the spinal cord as shown in the figure below (Figure 1-1). When the pain signals arrive at the spinal cord, they are in turn relayed to the higher centers of the brain--thalamus and cortex.

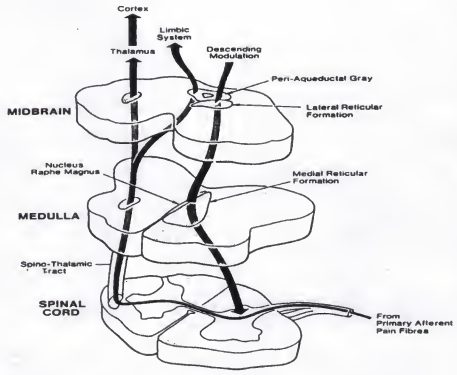


Figure 1-1 : The pain modulating system (adapted from Puntillo, 1988).

1.1.2 Pain Perception

Although some responses are reflexive in nature (e.g., knee jerk), the perception and appraisal of pain usually occurs in the higher centers of the brain. These systems are known to be responsible for attention, mood, motivation and arousal. Thus, pain is perceived in the thalamic and forebrain levels and evaluated in the cortex (Puntillo, 1988).

The perception and reaction to pain varies with each individual. It is now evident that a host of biochemical substances, including neurotransmitters and endogenous opioids, can modulate pain by either facilitating or inhibiting the transmission of pain impulses at various levels of the nervous system. There is evidence that the pain suppression system is mediated in part by endogenous opioids along the descending pathway, which relays processed information in response to the pain stimulus (Figure 1-1). The administration of exogenous opioids, like morphine and codeine, is thought to enhance this pain suppression system. However, the exact relationship between the analgesic effect of opioids and the role of pain modulators is yet to be clearly established.

1.2 Codeine

Codeine (Figure 1-2) is a naturally occurring alkaloid in which the phenolic hydroxyl group of morphine is replaced by a methoxy group. It was isolated in 1832 by Robiquet from the opium plant, *papaver somniferum*. Barbier in 1834 was the first to report its analgesic activity in humans (Baselt and Cravey, 1989).

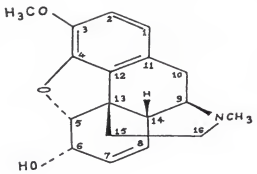


Figure 1-2 : The structure of codeine (adapted from Muhtadi and Hassan, 1981).

Codeine is a white crystalline powder which, when made anhydrous, melts at 154-156 ^{0}C. The phosphate salt is more soluble than the sulfate salt and hence it is used more commonly. The free base is sparingly soluble in water, but freely soluble in alcohol. Codeine is a monoacidic, weak base with a pKa value of 8.2 (Baselt and Cravey, 1989). It exhibits a characteristic UV absorbance peak in water at 284.8 nm (Grasselli and Ritchey, 1975). The anhydrous base has a nominal molecular weight of 300.

1.2.1 Administration and Dosage

Codeine is usually given orally as a phosphate or sulfate salt for the relief of cough and mild to moderate pain. The phosphate salt may also be given parentally for the relief of pain by intramuscular or subcutaneous injection.

As an analgesic, the usual oral dose is 30 to 60 mg every four or six hours, as needed for the relief of pain. For treatment of cough, the usual adult dose is 10 to 20 mg every four to six hours, not to exceed 120 mg. As with other

opiate agonists, the smallest effective dose must be given in order to minimize the development of tolerance and physical dependence.

1.2.2 Pharmacological Actions

Codeine, like morphine, acts by blocking excitatory synaptic transmission in the central nervous system and relieves pain and anxiety primarily by raising the pain threshold. It exerts a combination of depressing and stimulating effects on the central nervous system and various peripheral organs. Important CNS effects include analgesia, euphoria, sedation and respiratory depression. Supression of the cough reflex is a well-recognized action of opioids, particularly codeine. Miosis (constriction of pupils) is another pharmacological action seen with virtually all opioid agonists. Codeine can also cause activation of the brain stem chemoreceptor trigger zone to produce nausea and vomiting.

Peripheral effects of codeine include increasing the tone and decreasing the rhythmic contractions of different types of smooth muscles. In the gastrointestinal tract this produces constipation, which may be troublesome in ordinary analgesic therapy, but useful in the treatment of diarrhea (Jaffe and Martin, 1985). Urethral and biliary tract spasms are usually increased by codeine, but the analgesia produced may outweigh these undesirable effects. It can, however, be life threatening in cases of asthma when combined with repiratory depression.

Other effects of codeine include central vasomotor capacity depression and dilatation of some vessels, including the coronary arteries. On the whole, these circulatory effects are small and probably result from a combination of

central actions and peripheral histamine release. Some anticholinergic activity may be present, but is probably not critical (Way and Adler, 1962).

1.2.3 Toxicity

Codeine shares the toxic potentials of the opiate agonists. The most common side effects observed after the administration of therapeutic doses of codeine include dizziness, sedation, nausea, vomiting, sweating and a feeling of light-headedness. Other adverse effects that can be seen include euphoria, dysphoria, weakness, headache, insomnia, anorexia, gastrointestinal distress, bradycardia and even urinary retention.

Toxic effects of opioid overdose produce a classic triad of signs : coma, respiratory depression and constricted pupils. Breathing becomes shallow and irregular and may slow to as low as 2-4 breaths per minute. A severe overdose of codeine can cause respiratory depression, cyanosis, extreme somnolence which can progress to a coma, and severe hypotension with bradycardia. This could lead to apnea, circulatory collapse, cardiac failure and finally death. Codeine toxicity can be treated successfully with an intravenous administration of the narcotic antagonist naloxone (Cutting, 1972 ; Jaffe and Martin, 1985 ; McEvoy, 1990).

1.2.4 Therapeutic Uses

Codeine is a mild analgesic indicated for symptomatic relief of moderate pain. It is considered to be 1/10 to 1/6 as potent as morphine as an analgesic. It is also used as an antitussive, alone or in combination with other antitussives or

expectorants, in the symptomatic relief of non-productive cough (Cutting, 1972 ; Jaffe and Martin, 1985 ; McEvoy, 1990).

1.2.5 Drug Dependence and Tolerance

The major limitation of opioids is that they characteristically produce "drug habituation" or "addiction". Drug dependence is marked by tolerance--the gradual deveopment of resistance to the effects of the drug after repeated administration. Tolerance is manifested by a decline in the effectiveness of a drug, requiring a gradual increase in the dosage in order to maintain the initial effect.

Psychic dependence is a clinical term to indicate habituation. It is defined as compulsive use of a drug by an individual who feels euphoric and a sense of well-being from its chronic use. This kind of dependence is seen to a lesser or greater extent with numerous agents like caffeine, nicotine, salicylates and bromides as well as narcotic analgesics.

Physical dependence, on the other hand, deals with the biochemical and physiological adaptation of tissues to a new chemical environment after repeated use of a drug. The drug becomes necessary for normal tissue function and its withdrawal causes an abnormal cellular response referred to as "abstinence syndrome". This situation is usually characterized by effects opposite to those of the pharmacological effects of the drug.

The phenomenon of physical dependence can actually be visualized as being due to the prolonged occupation of the receptor sites within the cells of the central nervous system by opioid analgesics. This receptor-drug interaction leads to adaptive changes in the latent cellular excitability. These changes then manifest themselves during drug abstinence as symptoms of withdrawal. The

intensity of the withdrawal syndrome is proportional to the amount and duration of drug administration.

1.2.6 Analytical Techniques

Until recently there was little pharmacokinetic data described in the literature regarding low doses of codeine. This was mainly due to the lack of analytical techniques of sufficient sensitivity and specificity. Earlier studies relied on colorimetric assays which were not very sensitive (Woods, Muehlenbeck and Mellett, 1956). Johannesson and Woods (1964), Yeh and Woods (1969, 1970) used high doses of radiolabeled codeine and were able to measure codeine and biotransformed morphine in rat plasma.

As codeine undergoes extensive metabolism, forming active metabolites, there is a need to develop an assay to precisely determine the extent of formation of each metabolite and quantify its potential contribution to the overall analgesia and/or toxicity associated with codeine. The older analytical methods to determine the levels of codeine and some of its metabolites included radioimmunoassay (Findlay et al., 1977 ; Gintzler et al., 1976), gas chromatography (Jain et al., 1977 ; Kogan and Chedchel, 1976) and gas chromatography-mass spectroscopy (Ebbighausen et al., 1973 ; Cone et al. 1983) techniques.

Although radioimmunoassays offer the sensitivity required for the detection of these compounds, differentiation between very similar species like morphine 6-glucuronide and morphine 3-glucuronide cannot be achieved. The ability to identify and quantify the above metabolites is important, since morphine 6-glucuronide is known to be pharmacologically active. Gas chromatography and

mass spectroscopy can offer both sensitivity and specificity required for examination of codeine and morphine, but the techniques involve time-consuming derivatization steps and are not suitable for the glucuronides. Many researchers have turned their attention to the development of rapid, sensitive and specific HPLC methods for the detection of opiates.

Numerous HPLC-based methods have been reported with ultraviolet (Persson et al., 1989), fluorescence (Chen et al., 1989 ; Tsina et al., 1982) and electrochemical (Harris et al., 1988 ; Svensson 1986 ; Verway-van Wissen et al., 1991 ; Besner et al., 1989 and Bedford and White, 1985) detection systems. HPLC with fluorescence detection requires the conversion of some compounds to fluorescent products before analysis. Electrochemical detection does not allow simultaneous detection of all the compounds, due to differing redox potentials. HPLC with ultraviolet detection would therefore seem to be the method of choice for developing an assay for all of the compounds of interest.

A potential problem with using a reversed phase HPLC system is that the polar glucuronides elute very close to the solvent front and are prone to being hidden by co-eluting endogenous substances (Chari et al., 1991). An alternate method has been described for morphine and its metabolites using a normal phase system (Wielbo et al., 1993).

1.2.7 Pharmacokinetics in Man

There is an abundance of literature describing the pharmacokinetics of codeine in man (Quiding et al., 1986 ; Guay et al., 1987 ; Chen et al., 1991 ; Yue et al., 1989 a , 1990 a, b ; Shah and Mason, 1990 a ; Way and Adler, 1962 ; Findlay et al., 1977, 1978, 1986 ; Hull et al., 1982 ; Rogers et al., 1982 ; Persson

et al., 1992 ; Vree and Verway-van Wissen, 1992). Despite this extensive documentation, the relevance of some of the active metabolites of codeine is not clear. The extent of formation of individual metabolites and their potential contributions to the analgesic efficacy seen after codeine administration need to be assessed in detail.

1.2.7.1 Absorption

Codeine is well absorbed following oral and intramuscular administration in man. Its bioavailability after oral administration was found to be 50-60% (Rogers et al., 1982). At a dose of 60 mg, a peak plasma concentration (C_{max}) of around 100-200 ng/ml was seen within one to two hours after administration (Mohammed et al., 1993). Chen et al. (1991) observed that the mean t_{max} (time at which the peak plasma concentration is observed) for codeine occurred about 1 hour after oral administration in the case of both single and chronic dosing. However, the mean peak plasma concentration after chronic dosing was significantly higher than after single dosing. This was also observed by Quiding et al. (1986) and can in part explain why some subjects experience a greater analgesic effect after chronic dosing compared to single dosing.

1.2.7.2 Distribution

After the drug is absorbed into the blood, it is distributed to tissues in the body. However, only the free drug concentration can equilibrate with these tissues. The main interaction in blood is between the plasma proteins and the drug molecules and this is usually a reversible physical process. Thus, the binding of drugs to plasma proteins is a dynamic process. Codeine has been shown to be bound to plasma proteins to an extent of 25-30% (Findlay et al., 1977). Baselt and Cravey (1989) reported a volume of distribution of about

3.5 l/kg for codeine, indicating an extensive distribution in the various tissues of the body.

1.2.7.3 Metabolism

Codeine is primarily metabolized in the liver, the major site being the microsomes in the endoplasmic reticulum. Lesser, but significant sites include the central nervous system, kidney, lung and placenta. Metabolism is predominantly via conjugation with glucuronic acid at the 6-position (Yue et al., 1989 a, b, 1990 a, b ; Chen et al., 1991 ; Vree and Verway-van Wissen, 1992). Other metabolic pathways include O-demethylation to morphine and N-demethylation to norcodeine (Sindrup et al., 1990). These primary metabolites of codeine are further metabolized to their glucuronides as outlined in Figure 1-3.

The hepatic biotransformation of codeine to morphine has led many to believe that that codeine may exert its analgesic effect through partial conversion to morphine (Adler and Latham, 1950 ; Findlay et al., 1978 ; Yue et al., 1990 a). This assumption is supported by the low affinity of codeine to the μ opiate receptor and by the marked *in vivo* analgesic efficacy of morphine, morphine-6-glucuronide and normorphine (Lasagna and Kornfeld, 1958 ; Osborne et al., 1988). However, studies by Quiding et al. (1986) and Shah and Mason (1990 a) have questioned the possible role of morphine in codeine analgesia because of the very low concentrations of morphine seen after both single and repeated doses of codeine.

Chen et al. (1991) described codeine 6-glucuronide pharmacokinetics in detail after single and chronic oral codeine administration. There was no difference in the plasma t_{max} (1.28 versus 1.13 h) and C_{max} (1.43 versus 1.38 μg/ml) for single and chronic dosing, respectively. The mean AUCs for codeine

and codeine 6-glucuronide at steady state were not significantly different for either single or chronic dosing. The average ratio of the AUC_{C6G} : AUC_{Cod} was

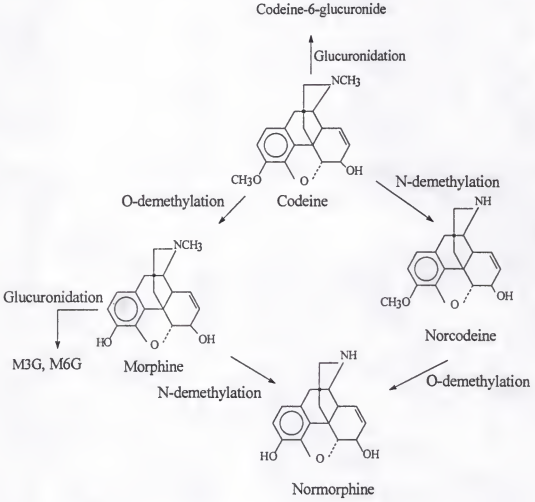


Figure 1-3 : The various metabolic pathways of codeine.

about 15 for both single and chronic dosing regimens. Vree and Verway-van Wissen (1992) reported similar values for the various pharmacokinetic parameters of codeine 6-glucuronide except in the case of $t_{1/2}$ and AUC ratio.

While they reported $t_{1/2}$ values for codeine and codeine 6-glucuronide as 1.5 and 2.8 hours respectively, Chen's group found the $t_{1/2}$ values for both compounds to be similar, about 3 hours. Vree's group reported a AUC_{C6G} : AUC_{Cod} ratio of 10 compared with 15 by Chen's group.

Quiding et al. (1986) reported plasma concentrations of morphine both after single and multiple doses of 60 mg of codeine to be about 2-3% of that of codeine. Shah and Mason (1990 a) found that Morphine AUC values ranged from 2-5% after a 60 mg oral dose of codeine. However, Vree and Verway-van Wissen (1992) reported that no free morphine could be detected in the plasma of human volunteers who took 30 mg of codeine orally. Any morphine formed was immediately glucuronidated at the 3- and 6- positions to form the corrosponding glucuronides. Both glucuronides were detected in the plasma with the morphine 3-glucuronide concentrations being higher than those of morphine 6-glucuronide. Very small amounts of normorphine, norcodeine and its glucuronide conjugate, norcodeine 6-glucuronide, were also detected in the plasma.

1.2.7.4 Elimination

Codeine and its metabolites are excreted almost exclusively by the kidneys. A very small fraction is eliminated as free codeine (about 5%). The major portion of the administered dose appearing in the urine consists of biotransformed products. Urinary recoveries of codeine and its metabolites indicate that codeine 6-glucuronide is the major metabolite formed from codeine. There are also trace amounts of morphine and its glucuronides along with normorphine and its glucuronide conjugate (Chen et al., 1991). The excretion of codeine and its metabolites in 24 hour urine as a% of the dose of administered codeine is summarized in Table 1-1. Adler et al. (1955) observed that, after a

single dose of codeine, urinary excretion was almost complete in 24 hours, although trace amounts of codeine and morphine stayed in the body for several days before being completely eliminated. A small percentage of the dose (0.02-0.17%), consisting mostly of free codeine and some metabolites, was also detected in the feces.

Codeine / Metabolites	% Excreted in 24 Hour Urine
Codeine	8 - 16
Morphine	0.5 - 1
Codeine 6-glucuronide	48 - 69
Morphine 6-glucuronide	0.5 - 2
Morphine 3-glucuronide	5 - 8
Norcodeine	2 - 10

Table 1-1 : The urinary excretion data in humans after a 60 mg oral dose of codeine. (from Yue et al., 1990 a).

Codeine has an elimination half-life of about 2-3 hours (Findlay et al., 1977, 1978 ; Quiding et al., 1986 ; Yue et al., 1990 a, b). Chen et al. (1991) reported that elimination half-lives for both codeine (3.2 versus 2.9 h) and codeine 6-glucuronide (3.2 vs 3.3 h) after both single and chronic dosing, respectively, were not significantly different. The renal clearance of codeine is between 67 and 265 ml/min. The creatinine clearance values of codeine in healthy volunteers has been reported to be in the range of 90 to 132 ml/min., indicating that in addition to glomerular filtration, codeine can undergo active secretion into the lumen of the proximal tubules (Chen et al., 1991).

1.2.8 Pharmacokinetics in Rats

The physiological disposition of codeine in various experimental animals after relatively high doses has been extensively studied. Studies done in male rats have shown that about half the dose (55%) of codeine undergoes O-demethylation (Yeh and Woods, 1969). Morphine formed by the metabolic conversion of codeine has been found in the plasma, urine, bile and feces (Johannesson and Shou, 1963 ; Johannesson and Woods, 1964 ; Yoshimura et al., 1970). Morphine has also been determined to be present in the brain of rats following large doses of codeine (Dahlstrom and Paalzow, 1976).

Traditionally, the major routes of administration in rats have been subcutaneous (s.c.) and intraperitoneal (i.p.) injections. Numerous pharmacological experiments with rats to determine analgesic activity of codeine, morphine (Johannesson and Shou, 1963 ; Yeh and Woods, 1969 , 1970 ; Oguri et al., 1990) and the 3- and 6-glucuronide metabolites (Yoshimura et al., 1973 ; Shimomura et al., 1971 ; Abbott and Palmour, 1988 ; Sullivan et al., 1989 ; Gong et al., 1991 ; Paul et al., 1989 ; Pasternak et al., 1987 ; Smith et al., 1990), have utilized both s.c. and i.p. routes of administration. There are a few reports in the literature regarding intravenous administration of codeine and morphine in rats (Dahlstrom and Paalzow, 1976 ; Shah and Mason, 1991 ; Bhargava and Villar, 1992 ; Thurston et al., 1993). Shah and Mason (1990 b) also administered codeine orally in rats as well as by i.v. injection and compared the two routes of administration.

1.2.8.1 Absorption

Shah and Mason (1990 b) described codeine pharmacokinetics after an oral dose of 5 mg/kg in rats. A solution of codeine phosphate was made by

dissolving it in 2-4 ml of physiological saline. The drug was then carefully delivered via gastric intubation to the fasting animals. Codeine was rapidly absorbed after the 5 mg/kg oral dose. The mean peak plasma concentrations were 101.3 $\pm$ 42.4 ng/ml around 6.4 $\pm$ 4.5 min after dosing. After 4 hours, no codeine could be detected in the whole blood with the HPLC method used in the study. A large intersubject variation was observed in the absorption of codeine. This variation may be partly explained by factors such as gastric motility and intestinal transit time.

The mean bioavailability was calculated from oral $AUC_0^* * D_{i.v.}$ / i.v. $AUC_0^* * D_{oral}$ as 0.08 $\pm$ 0.03, that is., only 8% of the ingested dose of codeine reaches the systemic circulation. Absorption of opiates in general is thought to occur by passive diffusion rather than by processes invoving energy expenditure (Christensen et al., 1984). Incomplete absorption was not considered to be a major factor as the amounts of free codeine found in the feces were negligible following oral administration.

1.2.8.2 Distribution

Miller and Elliot (1955) conducted several distribution experiments in the rat. They showed that codeine was well distributed, and capable of leaving the the blood and concentrating in parenchymatous tissues such as liver, kidney, lung, adrenal glands and brain. This is reflected in the large volume of distribution of the terminal portion ($V_{d\ area}$ = 5.1 l/kg), obtained from the concentration-time profile after intravenous codeine administration.

Codeine given i.v. appears to fit a two compartment body model with a rapid distribution and a short terminal elimination phase. This indicates that codeine is rapidly metabolized and /or excreted. Elimination rate from the central

compartment (0.0569 min^{-1}) is more than the terminal rate constant (β), due to distribution of codeine into the peripheral compartment. The mean ratio of 0.92 for K_{12} / K_{21} (ratio of the inetrcompartmental rate constants) shows approximately an equivalent distribution of codeine between the central and peripheral compartments. Linear pharmacokinetics was exhibited by codeine at i.v. doses of 1-4 mg/kg (Shah and Mason, 1990 b).

1.2.8.3 Metabolism

There are a few studies in the literature that examine the metabolism of codeine in rats. These were done mostly in the 1960s and were conducted with radioactive codeine. Plasma concentrations after a 2 mg/kg s.c. injection of codeine showed the presence of free codeine, free morphine and conjugated morphine (in the ratio of 10 : 3 : 1). No conjugated codeine was seen either in the plasma or brain (Yeh and Woods, 1969). The conjugated morphine was later characterized as morphine 3-glucuronide. It was reaffirmed that morphine 6-glucuronide and codeine 6-glucuronide were not formed in rats (Yeh and Woods, 1970). However, using thin layer chromatography, a Japanese group (Yoshimura, 1970) detected traces of codeine 6-glucuronide (0.2%) in the urine of rats, showing that a small amount of this metabolite can be formed in rats. This has been supported by Oguri et al. (1990) who used a specific HPLC method and found the urinary recovery of this compound to be about 1% (Table 1-2). Recent findings (Oguri et al., 1990 ; Lawrence et al., 1992) have shown that morphine 6-glucuronide could not be detected in plasma or urine of rats after codeine administration.

1.2.8.4 Elimination

In vivo codeine disposition studies using radiolabeled carbon-14 have shown that about 74% of the injected radioactivity is excreted as free codeine, free morphine and morphine conjugate via the pulmonary, biliary, intestinal and urinary routes in male rats (Yeh and Woods, 1969). Of this 74%, 20-40% is eliminated in the expired air as CO_2 via the pulmonary pathway.

Codeine / Metabolites	% Excreted in 24 Hour Urine
Codeine	1 - 2
Morphine	4 - 5
Codeine 6-glucuronide	0.1 - 0.3
Morphine 6-glucuronide	Not detected
Morphine 3-glucuronide	23 - 24
Norcodeine	Not detected

Table 1-2 : Urinary excretion data as a% of a dose of codeine (from Oguri et al., 1990).

A significant part of an i.v. dose of codeine has been shown to undergo enterohepatic recirculation to the extent of 10-30% (Walsh and Levine, 1975). It is known that codeine can decrease gastrointestinal tract motility, thereby increasing transit time of morphine glucuronide in the intestinal tract. This would then allow longer exposure to bacterial glucuronide hydrolysis which, in turn, would favor enterohepatic recycling. Yeh and Woods (1969), using radioactively-labeled tracers, reported the following amounts recovered from intact bile : free codeine (1.3%), free morphine (0.9%) and conjugated morphine (43.1%).

Oguri et al. (1990) saw a substantial interspecies difference in the metabolism of codeine (Table 1-3). This can influence considerably the nature and duration of the pharmacological and toxicological activities of codeine. The development of molecular aspects of gene evolution has been applied extensively to explain species differences seen in drug metabolism (Nebert and Gonzalez, 1987).

Codeine/ Metabolites	**Mouse** (n=25)	**Rat** (n=4)	**Guinea Pig** (n=4)	**Rabbit** (n=3)
Codeine	6.8 + 0.7	1.6 + 0.2	1.6 + 0.2	2.2 + 0.5
Morphine	0.8 + 0.2	4.3 + 0.4	0.2 + 0.1	1.3 + 0.3
Codeine 6-glucuronide	1.6 + 0.2	0.2 + 0.1	39.8 + 3.9	24.5 + 3.7
Morphine 6-glucuronide	nd	nd	0.7 + 0.1	1.9 + 0.3
Morphine 3-glucuronide	7.6 + 1.0	23.9 + 2.8	1.6 + 0.2	17.9 + 1.4
Norcodeine	9.0 + 0.8	nd	nd	nd

nd = not detected

Table 1-3 : Urinary excretion data (% of dose) 24 hours after codeine administration in various species (from Oguri et al., 1990).

1.3 Drug Glucuronidation

1.3.1 Overview

A major pathway for drug metabolism and excretion is the generation of water soluble glucuronide metabolites. Since many drugs exhibit structural features that allow conjugation without previous phase I reactions, glucuronidations are viewed as first line detoxification mechanisms. Most

glucuronides of drugs are considered to be inactive and rapidly eliminated. Therefore, glucuronide metabolites of drugs are often neglected in pharmacodynamic and pharmacokinetic studies and are not taken into account when evaluating drug effects.

The pharmacological and toxicological relevance of glucuronidation was pioneered by Dutton and is summarized in a comprehensive review by Kroemer and Klotz (1992). The general reaction scheme which describes the conjugation of glucuronic acid to various drugs in the presense of glucuronosyl transferase enzymes is shown in Figure 1-4. This reaction mediates the formation of ether, ester, thiolic, N- and C- glucuronides.

Uridine diphosphate glucuronosyltransferases (UGTs) are enzymes located in the endoplasmic reticulum and therefore form a part of the microsomal fraction. UGTs are generally 50 to 60 kD in size and span the entire membrane of the endoplasmic reticulum. There is a small C-terminal domain located in the cytoplasm with the active site directed toward the lumen of the endoplasmic reticulum.

1.3.2 Direct Pharmacological Activity

The best documented example of glucuronide activity is that of morphine. Morphine is conjugated to give morphine 3-glucuronide and morphine 6-glucuronide in the liver (Wahlstrom et al., 1988 ; Coughtrie et al., 1989). Shimomura et al. (1971) observed that morphine 6-glucuronide had a direct analgesic effect in the hot plate test. Subsequently, the same group demonstrated that both morphine 6-glucuronide and morphine 3-glucuronide can penetrate the blood brain barrier (Yoshimura et al., 1973). Using the tail flick

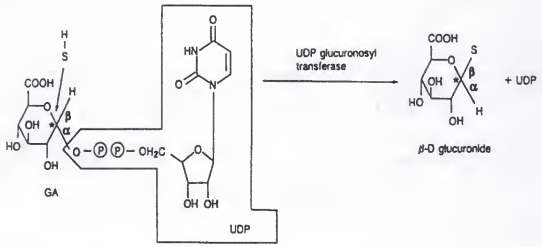


Figure 1-4 : Glucuronidation of a substrate (H-S) by reaction with uridine diphosphate-glucuronic acid (UDP-GA) in the presence of UDP-glucuronosyl transferase enzymes (from Kroemer and Klotz, 1992).

latency tests in rats, they found that morphine 6-glucuronide was 20-fold more potent than morphine after direct microinjection into the periaquaductal gray area of the brain. Morphine 3-glucuronide in this experimental design did not produce any effect.

The observations of Pasternak have been confirmed by a number of investigators (Paul et al., 1989), who performed detailed characterization of morphine 6-glucuronide. After peripheral administration to rats the analgesic effect was twice that of morphine itself. After intrathecal administration, morphine 6-glucuronide was reported to be 650 times more potent than the parent compound. Smith et al. (1990) showed that morphine 3-glucuronide had no analgesic activity but could act as a potent antagonist of morphine and morphine 6-glucuronide induced analgesia in rats. In this context Woolf (1981) reported that morphine 3-glucuronide was capable of inducing hyperalgesia in rats.

Recent investigations of force field and quatum mechanical characterization of morphine 3-glucuronide and morphine 6-glucuronide reveal an unexpectedly high degree of lipophilicity (Carrupt et al., 1991).

The question which then arises is whether this detailed pharmacological evidence for the contribution of glucuronides to the net drug effects of morphine is matched by clinical observations. Joel et al. (1985) speculated that morphine 6-glucuronide may contribute to the clinical efficacy of morphine. Hanks et al. (1987) tried to explain the potency of repeated oral doses of morphine as due to accumulation of morphine 6-glucuronide. Direct clinical evidence for the analgesic action of morphine 6-glucuronide was obtained by Osborne et al. (1988), who injected morphine 6-glucuronide, 1.0 mg/kg to 5 patients and 0.5 mg/kg to 1 patient. Five patients reported total pain relief within 30 minutes and the analgesia lasted for 1-7 hours. Pain and pain relief were monitored by visual analog scales (Figure 1-5).

Pharmacokinetic Parameters	**Patient A** (Normal Renal Function)	**Patient B** (Impaired Renal Function)
Clearance (l/kg) **	89	26
Volume of Distribution (l)	14.7	16.4
Elimination Half-life (h) **	1.9	7.4
Area Under the Curve (nmol $l^{-1}h$) **	370	1319

** indicates that the parameters were significantly different.

Table 1-4 : Pharmacokinetic parameters of two patients after a 1 mg/kg intravenous dose of morphine 6-glucuronide (from Osborne et al., 1988).

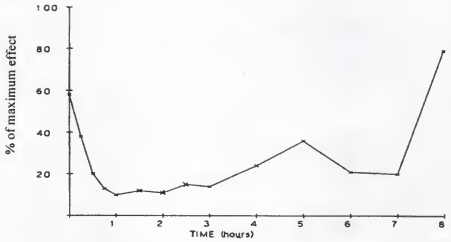


Figure 1-5 : A visual analog scale used for monitoring the extent of pain relief (from Osborne et al., 1988).

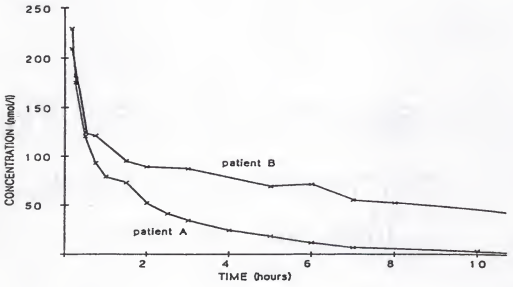


Figure 1-6 : The pharmacokinetic profile of two patients after 1 mg/kg i.v. administration of morphine 6-glucuronide (from Osborne et al., 1988).

Pharmacokinetic indices for two patients are shown in Table 1-4. Patient A had normal renal function while patient B had chronic renal impairment. The elimination of morphine 6-glucuronide was closely related to renal function. No morphine or morphine 3-glucuronide levels were detected in the plasma at any time. Plasma morphine 6-glucuronide levels for the two patients are shown in Figure 1-6.

Hannah et al. (1990) investigated the analgesic efficacy of intrathecal morphine 6-glucuronide in comparison with morphine in 3 patients with chronic cancer pain. The doses required for controlled analgesia were 393 $\pm$ 227 mg/24 h and and 227 $\pm$ 114 mg/24 h for morphine and morphine 6-glucuronide administration, respectively.

Conjugation of drugs by glucuronosyl transferases plays an important role in the overall picture of drug disposition. The resulting glucuronides represent metabolites that are not always inactive and may in fact contribute to drug action either directly (by producing analgesia as in the case of morphine 6-glucuronide) or indirectly (by release of the parent compound via hydrolysis as in enterohepatic recycling). Moreover, some glucuronic acid conjugates are not rapidly excreted. Their disposition can be modulated at different levels of distribution, metabolism and excretion, thereby modifying net drug action. Therefore, glucuronic acid conjugates should be taken into account when pharmacokinetic and pharmacodynamic characterization of drugs are determined.

1.4 Evaluation of Analgesia in Small Animals

1.4.1 Introduction

Eddy (1928, 1932) is credited as being the first to describe methods for determining analgesia in animal experiments by exerting a variable pressure on the distal part of the cat tail. Friend and Harris (1948) used a pair of forceps, whereby pressure could be exerted on the tail of the rat. Green et al. (1951) produced pain by exerting pressure on the tip of the tail using a syringe piston system.

Macht and Macht (1940) were the first to describe a method in which electrical stimulation was used to produce pain in rats. They implanted two electrodes in the skin of the scrotum. The rats reacted with a squeak response when the voltage was increased over a certain threshold. Luckner and Magun (1951) implanted two electrodes in the upper part of the tail. Collins et al. (1964) implanted an electrode in the rectum and another in the upper part of the tail in rats.

Eddy et al. (1950) placed mice in a cylindrical glass container, the base of which was a copper plate. This plate was maintained at a temperature of 50-55 ^{o}C by a hot water bath placed below the copper plate. The mice responded to the heat by licking their forepaws and trying to jump out of the cylinder. This technique is called Eddy's hot plate method and is a good pain model for the rat and mouse. D'Amour and Smith (1941) irradiated the tail tip with heat from a light source of defined strength. This tail flick method is also widely used for determinating analgesia in small animals.

A modification of the tail flick method was described by Berglund and Simpkins (1988). It involves measurement of the withdrawal time of the tail when a beam of light was focused on it. This was done both before drug administration, and at regular intervals thereafter. The former measurement was called baseline latency and the latter test latency. The instrument used was a model 33 tail flick analgesia meter (Iitc Inc., Landing, NJ, USA). and consisted of an incandescent light bulb with the beam intensity and sensitivity dial set at 75 and 8, respectively. The time between presentation of a focused beam of light and removal of the tail was recorded as the latency period. However, in the absence of any response, a cut-off period of 40 seconds was used to prevent tissue damage. This was considered as the maximal suppression of pain.

1.4.2 Time Course of Analgesic Effect

D'Amour and Smith (1941) were the first to determine the analgesic effect of morphine and codeine administered by i.p. injection. They found the effect to be maximal at 30 minutes post-injection for both drugs. Ercoli and Lewis (1945) injected equianalgesic doses of morphine and codeine, both intraperitoneally and subcutaneously. They found that both drugs had their greatest effect 30-60 minutes after administration, with the effect persisting for 60-120 minutes. Following higher doses, the degree of analgesia was found to be more complete.

Miller and Elliott (1955) were the first researchers to make a serious attempt to look into changes in the amounts of codeine and morphine in the brain with time. These investigators administered 25 mg/kg codeine-N-$^{14}CH_3$ by subcutaneous injection to rats. These rats were killed after 15, 30, 60 and 150 minutes respectively. The spinal cord and the brain were removed, and the

concentrations of these drugs were determined in the spinal cord, hypothalamus, cerebellum, medulla oblongata, mid-brain and parts of the hemispheres.

Miller and Elliott (1955) reported that concentrations of codeine rose sharply from the 15th to the 30th minute, while a slower rise was seen from the 30th to the 60th minute. The highest concentration of codeine was measured 60 minutes after drug administration. Another important fact to be considered is that relatively small amounts of both codeine and morphine enter the brain, and it is these concentrations which are responsible for producing analgesia. These results indicate a relationship between drug concentrations of codeine and morphine in the brain and the degree of analgesia produced. The exact relationship is yet to be firmly established.

1.5 Genetic Polymorphism

Genetic variation is an important cause of the large differences seen in drug metabolism between individuals. A number of isoenzymes in the cytochrome P450 family are involved in the oxidative metabolism of several essential drugs (Nebert et al., 1987). The entire population can be divided into extensive and poor hydroxylators based on the extent to which they metabolize certain drugs like mephenytoin, debrisoquine, sparteine and procainamide. It is well known that O-demethylation of codeine, leading to the formation of the analgesically active metabolites morphine and morphine-6-glucuronide, is catalyzed by cytochrome P450 IID6 isoenzyme (Nebert et al., 1989) and cosegregates with the debrisoquine/sparteine oxidative polymorphism .

Five to ten percent of caucasians are classified as "poor metabolizers" (PMs), since they lack the specific isoenzyme for O-demethylation (Yue et al.,

1990 a, b), while the remainder, capable of O-demethylating codeine, are termed "extensive metabolizers" (EMs). PMs are rare in the native Chinese population compared to Caucasians (Yue et al., 1989 a) indicating inter-ethnic differences in drug metabolism. This pharmacogenetic bias translates clinically into the fact that the PMs may not derive as much analgesic effect as the EMs. There are no data in the literature regarding genetic factors influencing the N-demethylation of codeine.

Glucuronidation reactions are catalyzed by a group of isoenzymes with overlapping specificities. Glucuronidation of codeine has been shown to be induced by smoking and oral contraceptives (Yue et al., 1990 c). It is also affected by the co-administration of other drugs like diazepam and chloramphenicol.

1.6 Immunomodulation

The immune process consists of a number of concerted events which include recognition and processing of foreign antigens, proliferation and differentiation of responder cells, and the production of proteins and peptides for the amplification and mediation of the immune response. In this regard, the immune system is not isolated and autonomous in nature. In fact it is a part of an interactive and communicative triad, that also includes the nervous and endocrine systems.

The immune system can affect brain functions as evidenced by the release of neurotransmitters and enhanced brain activity in certain localized regions after activation of the immune system following bacterial infection (Saphier, 1987). A number of centrally acting pharmacological agents have been

shown to affect immune function directly or indirectly via the hypothalamo-pituitary-adrenal (HPA) axis. Within this axis, feedback loops have been identified which, when activated by certain immune products such as cytokines, are responsible for the production of glucucorticoids, leading to marked immunomodulatory effects. These effects are enhanced in stressful situations (Herz, 1993). This is further indicative of the relationship between the brain and the immune system, either by neuronal pathways or by modulation of endocrine system

Drugs of abuse, particularly opioids, can produce deleterious effects on the immune system. From an immunological standpoint, it is necessary to determine whether the effects of drug abuse were due to the drugs, or the consequence of needle sharing and poor nutrition--both of which are frequently observed factors associated with drug addiction. Earlier epidemiological findings suggested that increased infections were caused by sharing of unsterilized and contaminated needles. However, subsequent clinical studies have focused on the increasing evidence that the opioids themselves are capable of affecting the host defense mechanisms by directly acting on the immune system.

1.6.1 Opioids Receptors

The concept of opioids as immunomodulators is based on studies showing the presence of classical opioid receptors on the surface of cells of the immune system. Ovadia et al. (1989) demonstrated that rat lymphocytic membranes possess a certain GTP binding protein that couples opioid receptors to adenylate cyclase in response to the action of an opioid on the lymphocytes. Wybran et al. (1979) reported an opioid involvement in immune function based

on their observations with human T lymphocytes. They indicated that the opioid-induced suppression of lymphocytes was reversible with naloxone.

Carr et al. (1994) found that naltrexone antagonized both the analgesic and immunosuppressive effects of mice, suggesting the involvement of the same receptor for both actions, that is, the μ receptor. This inference is supported by the findings of Ward et al. (1984) which showed that a μ-selective antagonist β-funaltrexamine, and not the δ-selective antagonist naltrindole (Portoghese et al., 1988) or the κ-selective antagonist nor-binaltorphimine (Portoghese et al., 1987), attenuated morphine-induced immune suppression.

1.6.2 Effects on Lymphocytes

Lymphocytes are the primary immunocytes responsible for cell-mediated (T lymphocytes) as well as humoral (B lymphocytes) immunity as seen in Figure 1-7. Measurement of the effect of various drugs on the proliferative capacity of lymphocytes is the most common *in vitro* assessment of functional cell-mediated responses. A large number of lymphocyte markers are suppressed following acute and/or chronic administration of opioids (Herz, 1993).

Naloxone-reversible reduction in the number of circulating lymphocytes in morphine-treated rabbits has been reported along with effects on the various subpopulations of lymphocytes (Herz, 1993). Arora et al. (1990) found an increase in the T helper to T suppressor ratio following morphine treatment. T lymphocyte rosette formation was one of the first functional measures of T cell function to be studied for comparing the immunosuppressive potential of various drugs. Wybran et al. (1979) showed that the suppressive effects of opioid agonists, in particular morphine, on the T cell rosette formation was

stereospecific for levorotatory forms, naloxone reversible, and produced tolerance.

Humoral immunity, on the other hand, involves antibody responses to drugs which are recognized as antigens by the immune system. Primary antibody responses were seen to be suppressed by the action of opioids in sheep erythrocytes (Lefkowitz and Chiang, 1975). Plaque-forming responses, which are indicative of antibody production, were suppressed in splenocytes obtained from mice implanted with morphine pellets (Bryant et al., 1990).

1.6.3 Effects on Myeloid Cells

Cells of myeloid origin include monocytes, macrophages, neutrophils, mast cells, basophils and eosinophils. In addition to serving as mediators of inflammatory responses, they are involved in numerous functions critical to early immune response. These functions include antigen presentation, antibody production, lysis of tumor cells, phagocytosis of foreign particles and the release of immune response mediators such as interferons, cytokines, transforming growth factor (TGF) and tumor necrosis factor (TNF).

In humans, morphine exposure leads to a depression of the phagocytic properties associated with myeloid cells (Tubaro et al., 1985). Macrophages, which are activated by the stimulatory agent γ-interferon, were inhibited in morphine-pelleted mice and the tumoricidal activity of the macrophages was seen to be completely abolished (Bryant et al., 1988 a). Chronic morphine administration in mice also inhibited macrophage colony formation as the result of a decreased expression of the macrophage stimulating factor (Herz, 1993).

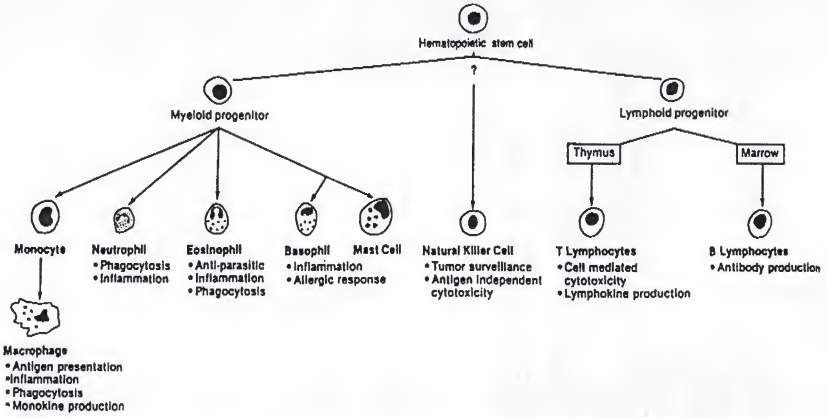


Figure 1-7 : The Classification and function of lymphoid cells (adapted from Herz, 1993).

1.6.4 Effects on Natural Killer Cells

Natural killer cells represent another type of immune cell and constitute only about 5% of the total leukocyte population. They are large cells possessing an intrinsic activity for non-specific killing and lysis of a variety of tumor cells. In addition to serving as scavengers of malignant cells, natural killer cells also play a regulatory role in antibody production.

Shavit et al. (1984) demonstrated that daily doses of subcutaneous morphine (50 mg/kg) suppressed natural killer cell tumoricidal activity (NK activity), and that naltrexone administration abolished this effect. This is strong evidence that the natural killer cytotoxic effect is centrally-mediated. Shavit et al. (1986 a) injected small quantities of morphine directly into the lateral ventricles and found that the NK activity was markedly suppressed. Novick et al. (1989) reported a marked decrease in NK activity in heroin addicts on methadone maintenance therapy.

NK activity is usually below normal basal levels in HIV patients and declines as the disease progresses. Endogenous opiate peptides, leucine-enkephalin and methionine-enkephalin have been reported to increase the cytolytic capacity of the natural killer cells (Wybran et al., 1987 ; Oleson and Johnson, 1989). This indicates that such candidates can potentiate the NK cell responses and restore immunocompetence in the case of pre-AIDS and AIDS patients.

1.6.5 Mechanism of Action

Opioids have been shown to down-regulate immune responses. The exact mechanism by which this immunosuppression occurs has yet to be

established. A direct mechanism of action is thought to be through lymphocyte opioid receptors (Figure 1-8). The second hypothesis is that the immune effects are indirectly mediated, either by the activation of the hypothalamic-adrenal-pituitary (HPA) axis with subsequent increase in the production of adrenal corticosteriods or by the release of catecholamines as a result of sympathetic innervation.

It has been observed that systemic administration of morphine suppresses the activity of NK cells in the rat (Shavit et al., 1984, 1986 a, b). The same group also reported that central administration of morphine produced the similar results, but required doses one third of those administered systemically. The NK suppression was blocked by naltrexone. N-Methyl morphine, a morphine analog which cannot cross the blood brain barrier, had no effect on the NK cytotoxicity when administered systemically. This is further evidence that the immunosuppressive effects of morphine are mediated by opioid receptors in the brain.

Morphine and other opioids are known activators of the HPA axis, and induce glucocorticoid output. Corticosterone has been implicated as possessing potential immunosuppressive effects as it was able to dose-dependently suppress NK activity *in vitro*. This effect was also observed in vivo in mice implanted with morphine pellets (Freier and Fuchs, 1994). A glucocorticoid receptor antagonist, RU 38486, blocked morphine-induced suppression of NK activity in a dose-dependent fashion. Naltrexone administration antagonized the morphine-induced elevation in serum corticosterone. This suggests that suppression of NK activity is linked to glucocorticoid elevation, which is the result

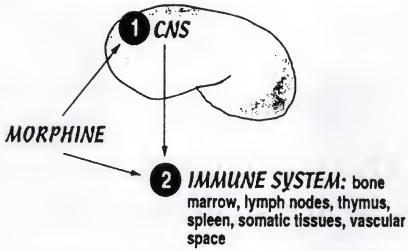


Figure 1-8 : The mechanisms of opioid-induced immunosuppression (from Peterson et al., 1990).

studies have shown that exposure of immune cells to opiates, especially morphine, results in a variety of functional disturbances (Chao et al., 1992, 1993; Peterson et al., 1991,1993).

There are many reports in the literature implicating opiates as immunomodulatory agents. Most of these studies have focused on morphine, the narcotic of choice in case of severe pain associated with trauma and cancer, and have shown that morphine possesses potent immunosuppressant activity. There are, however, no studies that investigate the immunosuppressive effects of codeine or any of the glucuronide metabolites of codeine and morphine.

A technique that simulates cellular immune response *in vitro* is the mixed lymphocyte reaction (MLR). It is based on the observation that lymphocytes from a mixture of genetically different individuals with different HLA (Human Leukocyte Antigen) types react with each other and proliferate. This test is performed by preventing the response of one set of lymphocytes (donor) through

radiation which then allows only the other set of lymphocytes (recipient) to proliferate as shown in Figure 1-9 .

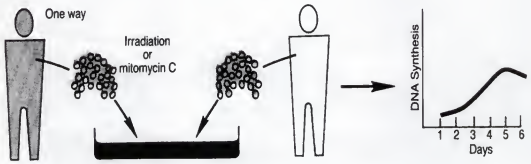


Figure 1-9 : A mixed lymphocyte reaction with cell proliferation.

Thus, opioids possess receptors which are capable of modifying immune functions. The concentrations achieved with analgesic doses of opioids are similar to those reported in the *in vitro* immunomodulatory experiments. It is also apparent that stress-induced activation of the endogenous opioid networks can contribute to various immunological changes. The focus for future research must include an understanding of the role of opioids in regulating immunity and their interaction with other immune function mediators.

1.7 Receptor Binding

In the case of morphinans, the aromatic ring and the basic nitrogen atom are necessary for analgesic activity. Substitutions at the phenolic hydroxyl group (position 3) and the alcoholic hydroxyl group (position 6) have been shown to cause pharmacological profiles which are opposite in nature. While additions at

the 6-position were seen to enhance opioid receptor binding affinities, changes at the 3-position significantly decreased receptor binding (Labella et al., 1979). The structure activity relationships of morphine and its 3- and 6-glucuronide metabolites have been evaluated in several studies (Yaksh et al., 1986 ; Gong et al., 1991 ; Shimomura et al., 1971 ; Pasternak et al., 1987).

On the basis of these results, investigators speculated that the inactivity of morphine 3-glucuronide compared with morphine 6-glucuronide could be due to the differences in the level of receptor binding. Christensen and Jorgensen (1987) showed that morphine 6-glucuronide, but not morphine 3-glucuronide, had a high affinity for the opiate receptors isolated from bovine brains in competition with ^{3}H-naloxone. Subsequent investigation by Pasternak et al. (1987) identified that morphine 6-glucuronide interacts with μ- but not κ-receptors.

The greater potency of morphine 6-glucuronide compared to morphine in antinociception studies has been reported by various investigators (Abbott and Palmour, 1988 ; Sullivan et al., 1989 ; Paul et al., 1989). In contrast to morphine and morphine 6-glucuronide analgesia, morphine 3-glucuronide produces hyperalgesia, respiratory stimulation and behavioral excitation by non-opioid mechanisms (Yaksh et al., 1986 ; Pelligrino et al., 1989). These studies suggest that morphine 3-glucuronide can actually antagonize the effects of both morphine and morphine 6-glucuronide. This is clinically important not only for the analgesic effect, but also for the respiratory depression associated with morphine administration.

1.8 Hypotheses

The hypotheses of this project are based on the overall aim of the project, that is, to examine and compare the analgesic and immunomodulatory effects of codeine and codeine 6-glucuronide.

1. Codeine 6-glucuronide, like morphine 6-glucuronide, possesses analgesic activity.
2. The glucuronide metabolites of codeine and morphine are less immunosuppressive than their parent compounds.

1.9 Specific Objectives

1. Develop and validate a reliable and sensitive HPLC-UV based assay for the quantitation of codeine, morphine, codeine 6-glucuronide, morphine 6-glucuronide and morphine 3-glucuronide in biological samples.
2. Chemically synthesize codeine 6-glucuronide utilizing a modification of the Koenigs-Knorr reaction.
3. Assess the immunomodulatory effects of codeine, morphine and their 6-glucuronide metabolites in human T lymphocytes (*in vitro*).
4. Compare the analgesic potencies of codeine and codeine 6-glucuronide in the rat using the tail flick method after intracerebroventricular (i.c.v.), subcutaneous (s.q.) and intravenous (i.v.) routes of administration.
5. Determine the μ opioid receptor binding affinities of codeine and codeine 6-glucuronide.
6. Analyze plasma and brain concentrations of codeine and their

metabolites at the peak analgesic response time after administration of codeine and codeine 6-glucuronide by various routes as described in specific objective #3.

CHAPTER 2
METHODS

2.1 Specific Objective #1: Analytical Method

An isocratic HPLC method was developed using a ultraviolet absorbance detector along with an efficient solid phase extraction method to analyze physiological concentrations of codeine, codeine 6-glucuronide, morphine, morphine 6-glucuronide and morphine 3-glucuronide in various biological samples, that is, human urine, rat plasma and rat brain. The method was first developed using blank human urine. It was then validated using rat plasma and brain samples.

2.1.1 Materials

HPLC grade methanol and acetonitrile were used (Fischer Scientific, Fairlawn, NJ, USA). Analytical grade potassium dihydrogen phosphate and 85% v/v o-phosphoric acid were supplied by Sigma (St. Louis, MO, USA) as were codeine, morphine, morphine 6-glucuronide and morphine 3-glucuronide. A sample of codeine 6-glucuronide was donated by the National Institute on Drug Abuse (NIDA, Rockville, MD, USA).

2.1.2 Extraction Procedure

2.1.2.1 Human urine

Solid phase extraction was performed with 3 ml Clean Screen® columns (Worldwide Monitoring, Horsham, CA, USA) containing 40 micron bonded silica

particles. The cartridges were placed on a 12 station Vac-Elut (Varian, Harbor City, CA, USA). The columns were conditioned with methanol (3 ml), distilled water (3 ml) and 0.025 M phosphate buffer pH 3 (1 ml). A 1 ml sample of urine mixed with 2 ml of phosphate buffer pH 3 was then loaded onto the column. The cartridges were air-dried for 30 seconds and then washed with 0.025 M phosphate buffer pH 3 (1 ml), followed by methanol (1 ml). The columns were air-dried again for 30 seconds before eluting the compounds with 3 ml of 5% freshly prepared ammoniacal methanol solution. The eluent was evaporated to dryness under a stream of nitrogen gas and the residue was reconstituted in 150 µl of the mobile phase and 50 µl was injected into the HPLC system.

2.1.2.2 Rat plasma

Extractions were performed with 10 ml Clean Screen® columns. The columns were conditioned with methanol (10 ml), distilled water (10 ml) and 0.025 M phosphate buffer pH 3 (2 ml). A 200 µL sample of rat plasma was mixed with 400 µL of 0.025 M phosphate buffer pH 3 and loaded onto the column. The cartridges were air-dried for 30 seconds and then washed with 0.05 M acetate buffer pH 4.5 (2 ml), followed by methanol (2 ml). The columns were air-dried for 30 seconds before eluting the compounds with 3 ml of 10% freshly prepared ammoniacal methanol solution. The eluent was evaporated to dryness under a stream of nitrogen gas. The residue was reconstituted in 150 µl of the mobile phase and 50 µl was injected into the HPLC system.

2.1.2.3 Rat brain

Brain samples were accurately weighed and an aliquot of 0.025 M phosphate buffer pH 3 (1 ml) was added to each sample. The samples were then homogenized and transferred to borosilicate tubes. After addition of a further 2

ml 0.025 M phosphate buffer pH 3, the samples were placed in a shaker for 10 minutes. The samples were then centrifuged at 4000 rpm for 20 minutes. The supernatant was removed and loaded onto 10 ml Clean Screen® columns. The columns were conditioned with methanol (10 ml), distilled water (10 ml) and 0.025 M phosphate buffer pH 3 (2 ml). After the samples were loaded, the cartridges were air-dried for 30 seconds and washed with 0.01 M acetate buffer pH 4.5 (2 ml) followed by methanol (2 ml). The columns were air-dried for 30 seconds before eluting the compounds with 3 ml of 10% freshly prepared ammoniacal methanol solution. The eluent was evaporated to dryness under a stream of nitrogen gas. The residue was reconstituted in 150 μl of the mobile phase and 50 μl was injected into the HPLC system.

2.1.3 Chromatographic Conditions

2.1.3.1 HPLC system 1

This system was used to examine human urine samples for the presence of opiates. It consisted of a Waters 501 multi-solvent pump set at a flow rate of 0.9 ml/min. The mobile phase consisted of 82% acetonitrile and 18% phosphate buffer (0.05 M potassium dihydrogen phosphate adjusted to a final pH of 3 with 85% v/v o-phosphoric acid). Separation of the compounds was achieved on a 20 cm x 4.5 mm I.D. Accubond® diol column with a 5 micron particle size (J & W Scientific Inc., Folsam, CA, USA). A Spectra-Physics Focus® multiwavelength forward optical scanning detector (San Jose, CA) set at 220, 230 and 280 nm was used to detect eluting compounds. Data acquisition and analysis was performed with Autolab® software loaded on a 386 IBM computer.

Apart from chromatographic analysis, the Autolab® software provided the option to examine the UV spectra of the compounds in the chromatograms. All of the compounds of interest exhibited a maxima in their UV spectra around 285 nm. A specific opiate of interest could be further characterized using derivative spectroscopy. In derivative spectroscopy, the first or higher derivative of absorbance with respect to wavelength is recorded versus wavelength. In this way the ability to detect and measure minor spectral features is considerably enhanced (Willard, 1986).

2.1.3.2 HPLC system 2

This system was used to determine concentrations of codeine, morphine and their glucuronides in rat plasma and brain samples. It consisted of a Waters 501 multi-solvent pump set at a flow rate of 0.7 ml/min. The mobile phase consisted of 88% acetonitrile and 12% phosphate buffer (0.05 M potassium dihydrogen phosphate adjusted to a final pH of 3 with 85% v/v o-phosphoric acid). Separation of the compounds was achieved on a 20 cm x 4.5 mm I.D. Accubond® diol column with a 5 micron particle size (J & W Scientific Inc., Folsam, CA, USA). Sample injection was automated by the use of a Waters™ 717 Plus autosampler. A Waters 486 tunable absorbance detector set at 220 nm was used to detect eluting compounds. A Millenium® 2010 Chromatography Manager software was used to acquire data. All the instruments were controlled by a 386 NEC Powermate computer, which also stored and processed the acquired chromatographic data.

2.2 Specific Objective #2: Synthesis of Codeine 6-glucuronide

Codeine 6-glucuronide is not available commercially. In order to evaluate its pharmacological activity in the rat, relatively large amounts of this compound (100-200 mg) were required. The National Institute of Drug Abuse (NIDA) is the only agency which provides samples of this compound, basically for analytical purposes (5 mg). For this reason, it was decided to synthesize codeine 6-glucuronide using a reproducible method described in the literature (Yoshimura et al., 1968). The initial attempt to synthesize codeine 6-glucuronide with some modifications of the Koenigs-Knorr reaction provided a good yield of the product, comparable to that reported in literature. A scheme of the synthetic route (Figure 2-1) is shown below.

2.2.1 Reaction Step I

In the first step, codeine monohydrate (1 g) was dissolved in 200 ml of dry benzene in a three-necked flask attached to a condenser with a drying tube containing drierite ($CaSO_4$) and a Dean-Starke trap. The trap was used to periodically distill benzene and to maintain anhydrous conditions in the flask. The solution was heated at 150 ^{0}C with an oil bath. Small amounts (250 mg) of freshly prepared silver carbonate along with equal portions of a solution containing 5 g of the acetobromo derivative of glucuronic acid, that is, methyl 2,3,4-tri-0-acetyl-1-bromo-1-deoxy-D-glucupyranuronate (obtained from NBS Biologicals Inc., Herts, UK), in 100 ml of dry benzene were added every hour over two seven hour periods.

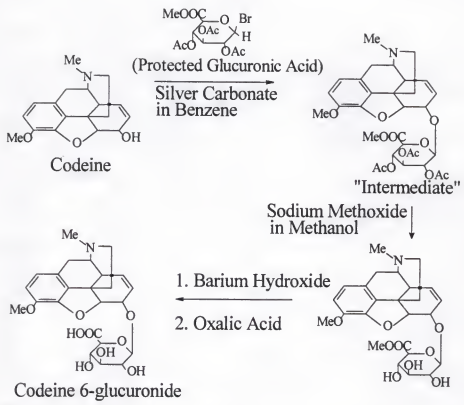


Figure 2-1 : Synthetic route of codeine-6-glucuronide using the Koenigs-Knorr reaction (adapted from Yoshimura et al., 1968).

Thin layer chromatography (TLC) was performed at regular intervals during the heating period to monitor the extent of the reaction. The solvent used for the TLC was a mixture of methanol and methylene chloride (1:4). Small portions (1 μl) were removed from the boiling mixture and spotted on TLC plates (Brinkman Polygram Sil G/UV 254). With increasing time, the spot representing the starting material codeine, disappeared and another spot with a higher R_f value appeared. This indicated the formation of a product assumed to be methyl [codein-6-yl 2,3,4-tri-0-acetyl-1-bromo-1-deoxy-D-glucupyranosiduronate, that is, the 6-glucuronide of codeine with intact acetyl and methyl groups on the glucuronic acid moiety, and subsequently referred to as the intermediate. The

structure and identity of the compound in $CDCl_3$ was confirmed by ^1H NMR (Varian EM 390 Spectrometer).

After the heating was stopped, the contents of the flask were filtered and the clear filtrate was evaporated to dryness with a rotory evaporator. The residue was redissolved in absolute ethanol and evaporated to dryness. The solid residue was then transferred to a column and chromatographed using 500 ml each of ethyl acetate, ethyl acetate : ethanol (60 : 40), ethanol : methanol (50 : 50) and methanol. Various fractions were collected and concentrated to dryness. The dried fractions which indicated the presence of the compound of interest were recrystallized using methanol. The melting point of the compound was 113-116°C, in agreement with the value previously reported (Yoshimura et al., 1968)

2.2.1.1 Dry benzene

A key point to guarantee the success of the first step was to ensure that the benzene used was absolutely dry. This was achieved by boiling benzene in the same apparatus as the reaction was done and removing the water with the Dean-Starke trap. Boiling for about 4 hours ensured removal of all the water present in the benzene.

2.2.1.2 Fresh silver carbonate

Freshly prepared silver carbonate is another essential prerequisite in the first step of the reaction, as the silver carbonate used as a catalyst is susceptible to oxidation in the presence of moisture and light. The procedure for preparing "active" silver carbonate (Wolfrom and Lineback, 1963) involves adding an aqueous solution of anhydrous sodium carbonate (1.6 g in 7.5 ml of distilled water) dropwise into a mechanically stirred solution of 8 g of silver nitrate dissolved in 20 ml of water. A solution of 1 g of anhydrous sodium hydrogen

carbonate in 12.5 ml of water was then added to the above in 2-3 portions. The mixture foams and a yellowish precipitate is formed. The solid recovered by filtration was silver carbonate.

2.2.2 Reaction Step II

The intermediate compound from step I (0.6 g) was suspended in a test tube with 3.5 ml of absolute methanol. A 1% solution of sodium methoxide in methanol (2 ml) was added and the mixture was stirred with a magnetic stirrer. The solution was evaporated to dryness *in vacuo*.

2.2.3 Reaction Step III

The dried residue from step II was dissolved in 2.2 ml of a 0.43N $Ba(OH)_2$ solution and stirred for about 4 hours before leaving it overnight in a refrigerator at 4^0 C. The barium salt which precipitated on cooling was dissolved in 4 ml of distilled water and adjusted to pH 6 with 2N oxalic acid solution. The solution was refrigerated overnight and the barium oxalate formed was removed by filtration. The filtrate was evaporated to dryness and the residue was recrystallized from methanol. The compound decomposed at 225-230 ^{0}C, which was slightly lower than that reported in the reference paper.

2.3 Specific Objective #3: Analgesic Activities of Codeine and Codeine 6-glucuronide

The polar glucuronide metabolites of codeine and morphine do not cross the blood brain barrier (BBB) as efficiently as their parent compounds. With this in mind, initial studies were designed to bypass the BBB and determine if

codeine 6-glucuronide produced analgesia in pain-induced rats by using standard antinociceptive tests, that is, tail flick and hot plate methods. This was done by delivering the compounds directly into the brain of rats via the intracerebroventricular route (i.c.v.). The next step was to compare the activities of codeine and codeine 6-glucuronide after subcutaneous and intravenous administrations using the same methods as in the i.c.v. studies for measuring the effects.

During the synthetic procedure, the intermediate compound formed after the first reaction step was isolated and characterized. This intermediate is the glucuronic acid moiety attached to the 6-position of codeine with intact acetyl/methyl groups. The antinociceptive activity of this intermediate was also investigated.

2.3.1. Intracerebroventricular Route Studies

These studies involved investigation of the antinociceptive effects of codeine, codeine 6-glucuronide and the intermediate compared to the standard analgesic drug, morphine. All drugs were dissolved in physiological saline (pH 4.5 - 5.5) and administered at doses of 100 µg/5 µl for codeine, 10 µg/5 µl for codeine 6-glucuronide and the intermediate, and a dose of 5 µg/5 µl for morphine. Each compound was administered to groups of 6 rats. One group of rats was injected with saline only and served as a control group. Another group was not injected with anything to control for any possible effects of the surgical procedure on the pharmacodynamic measurements. Measurements were made at the following time points after administration of the compounds : 0, 10, 20, 30, 40, 60, 90, 120, 150 and 180 minutes.

2.3.1.1 Surgery

After the animals were acquired, they were housed in cages in a room with a 12 hour light-dark cycle. The animals were fed on standard laboratory rat chow and tap water *ad libitum*. Each rat was anesthetized with 30-50 mg/kg sodium pentobarbital intraperitoneally and stereotaxically fitted with a 23 gauge intracerebroventricular stainless steel guide cannula. The co-ordinates used for the stereotaxic apparatus were : 1.0 mm lateral; 1.0 mm caudal to the bregma and 5.0 mm below the skull surface (Paxinos and Watson, 1986). The rats were allowed to recover for 3-5 days prior to starting the antinociceptive experiments.

2.3.1.2 Tail flick method

Analgesia was determined using the tail flick method of Berglund and Simpkins (1988) and previously described in section 1.4. Reduction in pain was expressed as the% of the maximum possible effect (% MPE) calculated as :

$$\% \text{ MPE} = \frac{(\text{ test latency - baseline latency })}{(\text{ cut-off period - baseline latency })} * 100$$

The area under the effect curve (AUEC) was determined from individual% MPE versus time graphs using trapezoidal calculation. The AUEC is considered a good indicator for comparing the intensity of effect during a certain time period. It also provides a good estimate of the duration of effect produced by each compound. Comparison of total AUEC values are, therefore, considered to reflect the relative effectiveness of the compounds.

2.3.2 Subcutaneous Route Studies

After the intracerebroventricular route, studies were performed by injecting the test compounds subcutaneously, that is, under the nape of the neck. Codeine, codeine 6-glucuronide and the intermediate were dissolved in physiological saline (pH 4.5 - 5.5) and injected at a dose of 10 mg/kg. A higher dose of 20 mg/kg of codeine was also administered to a group of 6 rats. The volume of injection was 1 ml/kg body weight of the rat. Each rat was also administered saline in a separate study and therefore, acted as its own control.

Assessment of the reduction in pain was determined by the tail flick method as described for the intracerebroventricular route. The intervals between time points for measuring the response were, however, greater than in the intracerebroventricular studies so as to take the absorption factor into consideration. The measurement time points were 0, 15, 30, 45, 60, 90, 120, 180, 240, 300 and 360 minutes.

2.3.3 Intravenous Route Studies

In these studies, the jugular vein of rats was catheterized so that compounds could be directly injected into the general blood circulation. Animals were first anesthetized with ketamine/xylazine (45:9 mg/100 g body weight of rat) intraperitoneally. A heparinized (100 U/ml) catheter (PE50 tubing, 0.58 mm X 0.965 mm) was then placed in the right jugular vein. The catheters were stoppered and exteriorized between the scapulae to avoid chewing. The rats were allowed 1-2 days to recover from the anesthesia and surgery. Drugs were dissolved in physiological saline (pH 4.5 - 5.5) and injected through the catheter. Codeine, codeine 6-glucuronide and the intermediate were administered at a

dose of 10 mg/kg. The injection volume was 1 mg/kg body weight of the rat. Physiological saline (500 µl) was also injected to ensure that the drug reached the systemic circulation and did not remain in the dead volume of the catheter.

As in the intracerebroventricular and subcutaneous studies, the tail flick method was used to determine the analgesic effect. As there was no absorption phenomenon to consider, periods between measurement times were less than in the subcutaneous studies but greater than the intracerebroventricular studies, that is, 0, 10, 20, 30, 40, 60, 90, 120, 180, 240 and 300 minutes.

2.3.4 Statistics

The area under the effect curve data for each compound was compared to its saline treatment using a paired Student's t-test. The area under the effect curve (AUEC) of compounds were also compared with each other using an unpaired Student's t-test.

2.4 Specific Objective #4: Immune Studies with Human T Lymphocytes

In vitro immune studies on the drugs were performed with human T lymphocytes found in peripheral blood mononuclear cells (PBMCs) which were isolated from the blood of healthy volunteers using the Ficoll-Hypaque density gradient centrifugation procedure. The isolated T lymphocytes were then stimulated by mitogens phytohemagglutin (PHA) and phorbol 12-myristate-13-acetate (PMA), activating the resting T cells and enabling them to become transformed into lymphoblast cells. These transformed cells are then capable of synthesizing DNA, dividing rapidly and proliferating.

When the lymphocytes reached their peak proliferation, they were labeled with 1 μCi ^{3}H thymidine after a period of 48 hours (for the PHA/PMA assay) and 120 hours (for the mixed lymphocyte reaction assay). The cells incorporated the radiolabeled thymidine into their DNA and counts were done using a scintillation counter. The differences in cell count determined with and without drugs was used as a measure of drug-induced changes in lymphocyte proliferation. Immunosuppression produced by drugs was expressed in terms of% inhibition of proliferation and calculated as :

$$\%\,\text{Inhibition of Proliferation} = \frac{(\text{CPM without drug} - \text{CPM with drug})}{(\text{CPM without drug})} * 100$$

2.4.1 Method

Peripheral blood (20 ml) was obtained from a healthy volunteer and transferred to a conical tube. To this conical tube 20 ml of RPMI tissue culture media containing 5% albumin was added (this culture media was developed at Rosewell Park Memorial Institute and hence the name, RPMI). A 10 ml Ficoll-Hypaque solution was drawn up in a pipette and carefully delivered to the bottom of the tube. The tube was then placed in a balanced centrifuge for 30 minutes at 1200 rpm. A white layer containing lymphocytes formed between the culture media and red cells. The lymphocyte cells were transferred into another conical tube and diluted with 50 ml of the RPMI cell culture media. The tube was centrifuged again for 10 minutes at 1200 rpm. A "pellet" of cells was seen to be formed at the bottom of the tube. The supernatant was decanted until 1 ml of the

media remained in the tube with the pellet. The tube was then slightly agitated to disperse the pellet homogeneously in the media and a cell-count was done to determine the total number of cells/ml using tryphan blue stain under a light microscope (Fischer Scientific, Fairlawn, NJ, USA) at a magnification of 40 X. The cell suspension was diluted with RPMI media to obtain a cell-count of $1x\ 10^6$ cells/ml for PMA and PHA assays and $2x10^6$ cells/ml for the MLR assay.

Stock solutions and dilutions of the drug to be tested were made in RPMI cell culture media. A 96-well U-bottom cell plate was used and 100 µl drug solutions of each concentration of each drug was pipetted into the plate in triplicate. To each drug-containing well was added, 100 µl of either PHA (5 µg/ml) or PMA (50 ng/ml) containing T lymphocytes. The controls in the assay consisted of T cells without the drug, T cells containing the mitogens (PHA or PMA) but without the drug, and a control with only the cell culture media. The cell plate was covered on the top and placed in an incubator until it was radiolabeled.

Standard drug dilutions were done in the same way as in the case of PHA/PMA assays. Lymphocytes from two separate individuals were used for this assay. The cells of one individual was selected to be irradiated and labeled as the donor. This was done in a cell irradiator with a ^{137}Cs source at 2500 rads for 4 minutes. To 100 µl of drug solution of varying concentrations, 50 µl of the irradiated cells and 50 µl of the plain cells from the other individual were added. The cell plate was covered and incubated until it was radiolabeled.

The cell plates in the PHA/PMA assay (on day 2) and MLR assay (day 5) were taken out of the incubator and placed on the work surface. ^3H-Thymidine (1 µCi) diluted with the RPMI culture media was taken out of the refrigerator. Using

a Hamilton microsyringe stored in ethanol, each cell well was given 10 µl of the ^{3}H-thymidine solution.

Cells were harvested onto nitromethylcellulose paper via a cell harvester attached to a vacuum pump. The cell plate was discarded in a radioactive waste box. Scintillation vials were set up and labeled appropriately. After the paper had dried, forceps were used to punch out individual circles (each representing a cell well) which were placed into the corresponding scintillation vials. Scintillation fluid (3 ml) was then added to each vial and the vials were capped tightly. Radioactivity counts were determined using a scintillation counter.

2.4.2 Statistics

The% inhibition of proliferation data of the compounds were compared to each other using an unpaired Student's t-test.

2.5 Specific Objective #5: Receptor Binding Studies

2.5.1 Materials

Tris-HCl, bovine serum albumin (BSA), morphine sulfate and naloxone HCl were obtained from Sigma Chemicals (St. Louis, MO, USA). Sodium chloride was purchased from Fischer Scientific Inc. (Fairlawn, NJ, USA). Codeine phosphate was obtained from Westlab Pharmacy, Gainesville, FL. Codeine 6-glucuronide and the intermediate were synthesized using the Keonigs-Knorr reaction. ^{3}H-DAGO ([D-ala^{2}, N-methyl-phe^{4}, glyol^{5}][tyrosyl-3,5-^{3}H] enkephalin) was used as a competitive ligand for the binding studies and was purchased from Amersham (Arlington Heights, IL).

2.5.2 Method

Receptor binding studies were carried out using brain tissues from Sprague-Dawley rats (200-300 g) using a modification of the procedure reported by Hochhaus et al. (1988). Brain tissue was homogenized in 60 volumes of 50 mM Tris-HCl buffer (pH 7.4) containing 100 mM sodium chloride. The homogenate was incubated in a water bath for 1 hour at 20 ^{0}C. After incubation, the homogenate was centrifuged at 20,000 rpm for 20 minutes at 4 ^{0}C. The pellet formed at the bottom was washed twice with 50 mM Tris-HCl buffer. The membrane suspension was vortexed for 2 minutes to ensure homogeneity.

Competitive binding studies were performed after adding 1% w/v of bovine serum albumin (BSA) to the membrane suspensions. The suspension (880 μl) was placed in polyethylene tubes and incubated with 20 μl of the tracer (1 nM ^{3}H-DAGO, a μ-receptor selective agonist) and 100 μl of the competing ligand (various concentrations of the drugs dissolved in 50 mM Tris-HCl buffer). This mixture was incubated in a water bath for 1 hour at 20 ^{0}C. After incubation, the bound and unbound fractions were separated by filtration using Whatman GF/C filters. The filter paper containing the retained radioactivity was transferred to scintillation vials and 3 ml of scintillation fluid was added to each vial. The vials were capped tightly and shaken to enable the radioactivity to be distributed in the scintillation cocktail. The vials were left overnight and tritium counts were performed using a liquid scintillation counter.

Specific binding of morphine, codeine, codeine 6-glucuronide and the intermediate to the μ-receptor was determined by competitive displacement of the radiolabeled tracer by various concentrations of the test compounds. The non-specific binding (NS) was determined with a relatively high concentration of

morphine (1 μM). The total binding (T) was determined from vials in which no drug was added. The counts per minute (CPMs) obtained from a scintillation counter were then plotted versus increasing drug concentrations. The data was fitted using an E_{max} model from the Scientist program (Micromath Scientific Inc., Salt Lake City, UT) as described in Equation 1. The raw data (CPMs) for each compound were standardized by transforming them into% of total binding and plotting them as a function of increasing drug concentration (Equation 2). The equations used for fitting the data are as follows :

Equation 1 :

$$CPM = T - \frac{T * C^N}{IC50^N + C^N} + NS$$

Equation 2 :

$$\% \text{ Total Binding} = \frac{CPM}{T} * 100$$

2.6 Specific Objective #6: Plasma and Brain Concentrations

The objective of this study was to determine plasma and brain concentrations at the peak analgesic response time in the rat. This was achieved

by the administration of codeine or codeine 6-glucuronide to groups of rats (n=6) by intracerebroventricular, subcutaneous and intravenous routes (described in section 2.3). The peak response time from the tail flick experiments after each route of administration was used as the time at which plasma and brain samples were collected.

Rats were first anesthetized with metofane 5 minutes prior to the peak response time. At the appropriate time, the rats were decapitated using a guillotine. Trunk blood samples were collected in vacutainers containing EDTA as an anticoagulant. Plasma was obtained by centrifuging the blood at 2500 rpm for 20 minutes. Brain samples were obtained after removing the skull and other membranes attached to the brain. Both the plasma and brain samples were stored in a freezer at -80 ^{0}C. The samples were analyzed using the HPLC method described in section 2.1.

CHAPTER 3
RESULTS

3.1 HPLC Development

An HPLC-UV based method was successfully developed for the analysis of codeine, morphine, codeine 6-glucuronide, morphine 6-glucuronide and morphine 3- glucuronide in biological samples, that is, human urine, rat plasma and rat brain. A typical chromatogram obtained after injection of a standard solution containing 100 ng/ml of each compound is represented in Figure 3-1 (system 1). This system uses a multiwavelength scanning UV detector and allows the simultaneous examination of the same chromatogram at different wavelengths in the scanning range (Figure 3-2).

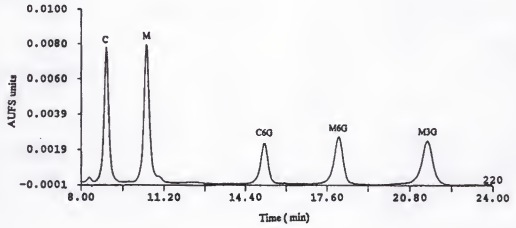


Figure 3-1 : A standard solution containing 100 ng/ml of each of all the compounds using system 1.

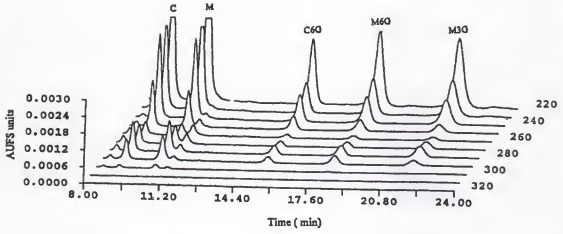


Figure 3-2 : The same chromatogram as in Figure 3-1 showing the multiwavelength capabilities of the detector.

The software also allows examination of the UV spectra at any time point in the acquired chromatogram (Figure 3-3). This can be used to identify the presence of an opiate, since all opiates exhibit a UV maxima of 285 nm. The differential UV spectra further helps to confirm the identity an opiate of interest (Figure 3-4). This is done by following minute changes in the spectra of an opiate and matching it with the spectra obtained from a standard solution of the same opiate.

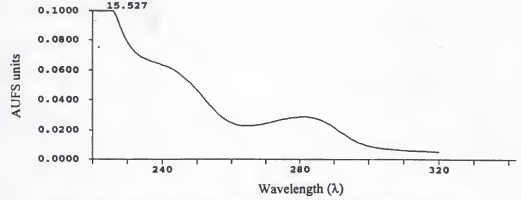


Figure 3-3 : A typical UV spectra which is exhibited by all opiates.

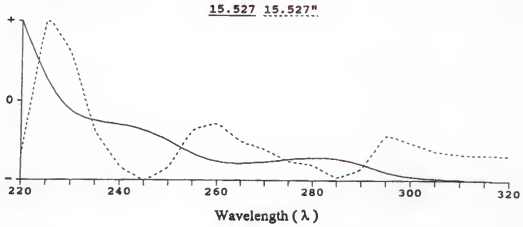


Figure 3-4 : A normal (—) and second derivative (---) spectra of morphine.

3.1.1 Extraction Recoveries

Extraction recoveries for each compound were determined by comparing the peak area of an extracted standard to an unextracted standard. The elution solvent of methylene chloride-isopropanol-ammonium hydroxide recommended by Worldwide Monitoring for opiates gave good recoveries for codeine and morphine only, but not for the glucuronide metabolites. However, the use of a 5% ammonium hydroxide solution in methanol enabled the efficiency of extraction of polar glucuronides from human urine samples to be increased. This elution solvent gave clean extracts and excellent recoveries in excess of 80% for all the compounds of interest. The% extraction recoveries for the various compounds of interest in human urine are represented in Table 3-1.

3.1.2 Range / Linearity of Standard Curve

After the initial development of the chromatographic system and extraction procedure, calibration curves were prepared in drug-free human plasma and

urine. Standards were set up for each compound of interest in the biological matrix (plasma/urine) at concentrations of 0, 10, 50, 100, 200, 400 and 500 ng/ml. Each point on the calibration curve was taken as the average of two determinations. The standard curve was determined from calibrators by the linear least squares fit to the equation y = mx + b, where x = concentration, y = drug peak area, m = slope of the line and b = y-intercept of the line. Linear correlations between the area under the peak and concentration of each compound was performed by regression analysis using the Microsoft Excel program. It was seen that each compound of interest had a good correlation with an R^2 of 0.99 or better over the range 0-500 ng/ml (Figures 3-5 and 3-6).

Compound	% Extraction recovery ± sd (n=4)
Codeine	92 ± 6
Morphine	90 ± 8
Codeine 6-glucuronide	86 ± 4
Morphine 6-glucuronide	85 ± 8
Morphine 3-glucuronide	83 ± 6

Table 3-1 :% extraction recoveries for compounds of interest in human urine.

3.1.3 Specificity

Drug-free plasma and urine samples were analyzed with and without the drugs. This was performed to show that the drug peaks were well separated from

the solvent front and did not have any interference from endogenous materials also eluting from the column.

3.1.4 Sensitivity/Limit of Detection and Quantitation

The detection limit was determined as the concentration of the sample which corresponds to the signal that is twice that of the baseline noise. When extracted from human urine using Clean Screen® columns, the minimum quantifiable concentrations of the various compounds using system 1 were : 5 ng/ml for codeine and morphine; 10 ng/ml for codeine 6-glucuronide, morphine 6-glucuronide and morphine 3-glucuronide.

3.1.5 Precision and Accuracy

Precision is defined as a measure of the closeness between replicate concentrations of a sample and its mean value. It is expressed as the% relative standard deviation about the mean (standard deviation/mean *100). Replicate (n = 7) analysis of the samples were done on the same day (intra-day) and over a period of two weeks (inter-day). Both intra-day and inter-day precision were determined to be less than 10% for all the compounds. The intra-day and inter-day precision values for codeine are shown in Table 3-2. Accuracy is expressed as the difference between the actual value and the mean measured value for each concentration. Accuracy determined from calibration graphs was less than 10%.

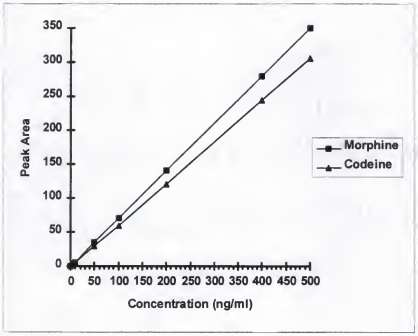


Figure 3-5 : A typical calibration curve for codeine and morphine in spiked urine.

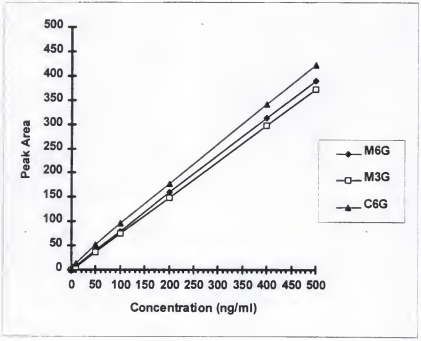


Figure 3-6 : A typical calibration curve for codeine 6-glucuronide, morphine 6-glucuronide and morphine 3-glucuronide in spiked urine.

3.1.6 Stability

Stability of extracted plasma, urine and brain samples were determined over a 24 hour period by replicate analyses during the assay development and validation. The variation in peak areas of samples determined from replicate analysis was less than 10%.

Concentration (ng/ml)	Intra-day variability (%)	Inter-day variability (%)
10	6.4	9.7
50	3.3	5.8
100	4.1	5.3
200	4.3	6.7
400	4.5	7.2
500	6.2	8.7

Table 3-2 : Intra-day and inter-day variability from replicate analysis of standards containing various concentrations of codeine.

3.2 Synthesis

In the first step of the synthetic procedure, the key to the success of the reaction was maintaining anhydrous conditions in the flask. This was achieved by using a Dean-Starke trap, which allowed distillation of benzene and water at regular intervals. The completion of the reaction was determined by the disappearance of the starting material by thin layer chromatography. The structure and identity of the compound formed after the first step was confirmed

by ^{1}H NMR. It indicated the presence of acetyl and methyl groups and the attachment of a glucuronic acid group to codeine. The melting point of the compound was 113-116^{0}C in agreement with the value reported by Yoshimura et al. (1968). The yield of this reaction was 70%.

Codeine 6-glucuronide was recrystallized from methanol and decomposed at 225-230 ^{0}C, slightly lower than the value reported by Yoshimura et al. (1968). This may be due to the fact that the product was apparently anhydrous compared to the half-hydrate product reported in the reference paper. Absolute methanol was used to recrystallize the compound instead of a water-methanol mixture used in the reference. The identity of the product was confirmed by comparing the retention time of the chromatographic peak to an analytical standard by using the HPLC method previously described (section 2.1). The overall yield of the reaction was about 16%.

3.3 Analgesia Studies

3.3.1 Intracerebroventricular Route

Intracerebroventricular administration of morphine, codeine, codeine 6-glucuronide and the intermediate produced significant antinociceptive responses in the rats. All the compounds tested produced a peak response about 20 minutes after administration. Each rat was also administered saline which produced minimal changes to baseline responses. The data set for the effect of surgery on the response time and the analgesic responses are summarized in Tables 3-3 and 3-4, respectively. It was observed that the surgical procedure

itself had no effect on the response time (Figure 3-7) and the analgesic response (Figure 3-8) and over a 3 hour period.

Time(min)	1	2	3	4	5	Mean	SD	SEM
0	11.8	8.7	8.1	7.8	8.4	9.0	1.6	0.7
10	11.5	6.8	9.5	11.2	8.1	9.4	2.0	0.9
20	9.2	7.0	10.3	9.6	10.3	9.3	1.4	0.6
30	7.2	8.5	8.2	11.1	11.8	9.4	2.0	0.9
40	7.4	9.4	9.6	8.8	11.3	9.3	1.4	0.6
60	12.2	12.2	11.4	9.6	10.8	11.2	1.1	0.5
90	9.7	9.1	10.3	9.3	11.1	9.9	0.8	0.4
120	9.0	11.2	10.8	8.4	9.4	9.8	1.2	0.5
150	10.8	9.6	11.8	8.7	9.7	10.1	1.2	0.5
180	11.3	9.2	11.6	9.2	10.1	10.3	1.1	0.5

Table 3-3 : The data for the effect of intracerebroventricular surgery on the response time (in seconds).

Time(min)	1	2	3	4	5	Mean	SD	SEM
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10	-1.1	-6.1	4.4	10.6	-0.9	1.4	6.3	2.8
20	-9.2	-5.4	6.9	5.6	6.0	0.8	7.5	3.4
30	-16.3	-0.6	0.3	10.2	10.8	0.9	11.0	4.9
40	-15.6	2.2	4.7	3.1	9.2	0.7	9.5	4.3
60	1.4	11.2	10.3	5.6	7.6	7.2	3.9	1.8
90	-7.4	1.3	6.9	4.7	8.5	2.8	6.3	2.8
120	-9.9	8.0	8.5	1.9	3.2	2.3	7.4	3.3
150	-3.5	2.9	11.6	2.8	4.1	3.6	5.4	2.4
180	-1.8	1.6	11.0	4.3	5.4	4.1	4.7	2.1

Table 3-4 : The data for the effect of intracerebroventricular surgery on the analgesic responses (in% MPE).

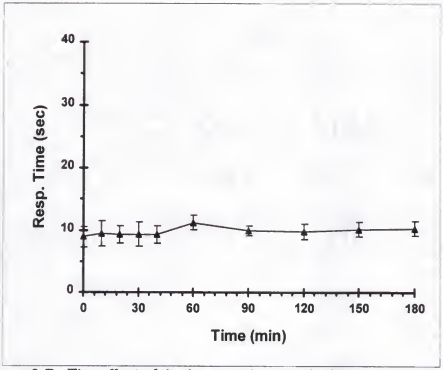


Figure 3-7 : The effect of the intracerebroventricular surgical procedure on the response time.

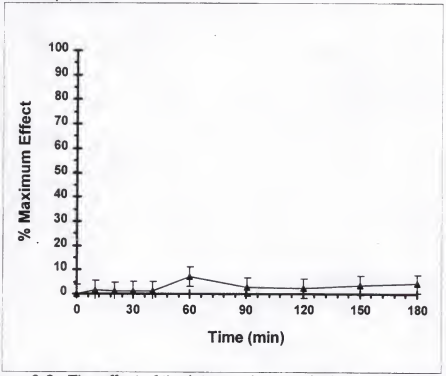


Figure 3-8 : The effect of the intracerebroventricular surgical procedure on the analgesic response.

The response time (Tables 3-5 to 3-9) for all the treatments was transformed into% of maximum effect data (Tables 3-10 to 3-14). The average values from the data sets were then plotted as a function of time (Figures 3-9 and 3-10). Morphine was the most effective of the compounds, producing a maximal suppression of pain, that is, 100% of the maximum possible effect (MPE) in the rats. Codeine 6-glucuronide and the intermediate also exhibited marked increases in antinociceptive responses, that is, up to 89 $\pm$ 6 and 81 $\pm$ 9% of MPE ($\pm$ standard error of the mean or SEM), respectively. Codeine also produced analgesia, with a peak effect of 63 $\pm$ 2% of MPE. The% MPE at peak response time for all the compounds is summarized in Table 3-15. The area under the effect curve (AUEC) was determined from individual% MPE versus time graphs for each treatment using trapezoidal calculation (Figure 3-11) and is summarized in (Table 3-16).

3.3.2 Subcutaneous Route

Significant responses were seen with codeine, but not with codeine 6-glucuronide or the intermediate after subcutaneous administration. There was also a difference in the peak response time. While codeine produced a peak response 30 minutes after administration, codeine 6-glucuronide and the intermediate showed a peak response 45 minutes after they were administered. The response time (Tables 3-17 to 3-20) for all the treatments was transformed into% of maximum effect data (Tables 3-21 to 3-24). The average values from the data sets were then plotted as a function of time (Figures 3-12 and 3-13). Codeine exhibited a significant response, that is, 61 $\pm$ 5% of the MPE (Figure 3-

13). On the other hand, codeine 6-glucuronide and the intermediate produced very poor responses, that is, 17 ± 3 and 11 ± 2% of MPE, respectively. The%

Time (min)	1	2	3	4	5	6	MEAN	SD	SEM
0	10.7	10.4	10.4	13.3	11.9	13.1	11.6	1.3	0.5
10	28.1	18.9	18.8	30.6	26.3	18.6	23.6	5.4	2.2
20	33.3	40.0	38.1	40.0	38.9	31.6	37.0	3.6	1.5
30	29.2	40.0	33.3	40.0	37.2	29.7	34.9	4.9	2.0
40	24.8	40.0	28.3	36.6	31.8	23.6	30.9	6.5	2.7
60	22.6	33.7	24.2	32.7	27.7	21.7	27.1	5.2	2.1
90	15.2	27.6	22.7	30.5	26.9	18.6	23.6	5.8	2.4
120	13.3	24.4	20.9	26.9	24.8	18.0	21.4	5.1	2.1
150	13.4	20.1	19.7	17.7	19.9	17.2	18.0	2.6	1.0
180	12.1	11.3	16.8	15.1	12.6	16.6	14.1	2.4	1.0

Table 3-5 : The data for the effect of intracerebroventricular administration of 10 μg of codeine 6-glucuronide on the response time (in seconds).

Time (min)	1	2	3	4	5	6	MEAN	SD	SEM
0	9.8	9.4	11.3	8.3	12.1	9.4	10.1	1.4	0.6
10	11.1	16.3	16.4	15.8	18.4	16.6	15.8	2.5	1.0
20	28.8	27.6	28.4	29.7	29.3	29.7	28.9	0.8	0.3
30	24.3	25.9	26.7	27.8	27.1	26.8	26.4	1.2	0.5
40	21.5	22.7	23.6	24.4	25.2	24.5	23.7	1.4	0.6
60	18.8	21.1	19.5	21.1	22.1	23.7	21.1	1.8	0.7
90	16.6	19.8	16.9	17.9	18.6	20.9	18.5	1.7	0.7
120	15.1	16.5	15.5	14.2	16.8	19.6	16.3	1.9	0.8
150	13.6	11.4	12.1	11.7	15.9	16.2	13.5	2.1	0.9
180	10.1	9.2	10.7	8.4	13.7	11.3	10.6	1.9	0.8

Table 3-6 : The data for the effect of intracerebroventricular administration of 100 μg of codeine on the response time (in seconds).

Time (min)	1	2	3	4	5	6	MEAN	SD	SEM
0	14.4	11.4	14.2	10.0	12.9	12.7	12.6	1.7	0.7
10	40.0	14.6	22.3	33.1	19.7	22.6	25.4	9.4	3.8
20	40.0	28.1	40.0	40.0	28.1	32.8	34.8	5.9	2.4
30	40.0	24.4	36.4	40.0	27.3	30.7	33.1	6.7	2.7
40	28.3	19.3	28.8	40.0	21.0	24.6	27.0	7.4	3.0
60	18.1	19.8	24.9	40.0	20.3	20.4	23.9	8.2	3.3
90	18.6	15.3	21.7	29.3	17.7	17.8	20.1	5.0	2.0
120	14.7	11.1	19.2	27.8	16.9	16.3	17.7	5.6	2.3
150	13.6	10.7	18.6	22.7	15.8	14.7	16.0	4.2	1.7
180	12.8	9.8	16.4	14.6	13.2	12.3	13.2	2.2	0.9

Table 3-7 : The data for the effect of intracerebroventricular administration of 10 μg of the intermediate on the response time (in seconds).

Time (min)	1	2	3	4	5	6	MEAN	SD	SEM
0	11.2	12.6	12.3	12.6	8.7	9.3	11.1	1.7	0.7
10	30.2	33.6	24.2	27.3	26.9	21.7	27.3	4.2	1.7
20	40.0	40.0	40.0	40.0	40.0	40.0	40.0	0.0	0.0
30	40.0	40.0	40.0	40.0	40.0	40.0	40.0	0.0	0.0
40	40.0	40.0	40.0	40.0	40.0	38.7	39.8	0.5	0.2
60	33.6	40.0	40.0	40.0	40.0	40.0	38.9	2.6	1.1
90	24.7	40.0	40.0	36.6	34.2	40.0	35.9	6.0	2.4
120	18.9	28.9	26.7	29.1	27.7	27.3	26.4	3.8	1.6
150	18.6	19.7	20.1	20.6	19.9	22.1	20.2	1.2	0.5
180	13.8	16.6	14.3	14.7	12.3	13.4	14.2	1.4	0.6

Table 3-8 : The data for the effect of intracerebroventricular administration of 5 μg of morphine on the response time (in seconds).

Time (min)	1	2	3	4	5	6	MEAN	SD	SEM
0	11.2	10.4	10.3	11.0	11.4	8.7	10.5	1.0	0.4
10	10.6	11.7	11.2	12.8	13.1	9.9	11.6	1.2	0.5
20	12.1	12.3	12.3	13.2	13.6	10.2	12.3	1.2	0.5
30	14.4	11.6	14.3	12.6	12.9	10.9	12.8	1.4	0.6
40	15.2	10.9	14.8	13.9	13.9	11.3	13.3	1.8	0.7
60	14.1	11.1	12.5	14.1	12.6	10.6	12.5	1.5	0.6
90	12.9	10.8	13.8	12.7	13.1	9.7	12.2	1.6	0.6
120	11.4	11.2	14.1	13.6	13.6	11.3	12.5	1.4	0.6
150	11.0	11.6	12.2	12.1	13.4	11.5	12.0	0.8	0.3
180	10.5	10.5	9.8	11.2	13.3	10.8	11.0	1.2	0.5

Table 3-9 : The data for the effect of intracerebroventricular administration of saline on the response time (in seconds).

Time (min)	1	2	3	4	5	6	MEAN	SD	SEM
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10	4.3	22.5	17.8	23.7	22.6	23.5	19.1	7.6	3.1
20	62.9	59.5	59.6	67.5	61.6	66.3	62.9	3.4	1.4
30	48.0	53.9	53.7	61.5	53.8	56.9	54.6	4.4	1.8
40	38.7	43.5	42.9	50.8	47.0	49.3	45.4	4.5	1.8
60	29.8	38.2	28.6	40.4	35.8	46.7	36.6	6.8	2.8
90	22.5	34.0	19.5	30.3	23.3	37.6	27.9	7.2	2.9
120	17.5	23.2	14.6	18.6	16.8	33.3	20.7	6.8	2.8
150	12.6	6.5	2.8	10.7	13.6	22.2	11.4	6.7	2.7
180	1.0	-0.7	-2.1	0.3	5.7	6.2	1.8	3.4	1.4

Table 3-10 : The data for the effect of intracerebroventricular administration of 100 μg of codeine on the analgesic responses (in% MPE).

Time (min)	1	2	3	4	5	6	Mean	SD	SEM
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10	59.4	28.7	28.4	64.8	51.2	20.4	42.2	18.6	7.6
20	77.1	100.0	93.6	100.0	96.1	68.8	89.3	13.1	5.4
30	63.1	100.0	77.4	100.0	90.0	61.7	82.0	17.3	7.1
40	48.1	100.0	60.5	87.3	70.8	39.0	67.6	23.2	9.5
60	40.6	78.7	46.6	72.7	56.2	32.0	54.5	18.3	7.5
90	15.4	58.1	41.6	64.4	53.4	20.4	42.2	20.3	8.3
120	8.9	47.3	35.5	50.9	45.9	18.2	34.5	17.2	7.0
150	9.2	32.8	31.4	16.5	28.5	15.2	22.3	9.9	4.0
180	4.8	3.0	21.6	6.7	2.5	13.0	8.6	7.4	3.0

Table 3-11 : The data for the effect of intracerebroventricular administration of 10 μg of codeine 6-glucuronide on the analgesic responses (in% MPE).

Time (min)	1	2	3	4	5	6	Mean	SD	SEM
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10	66.0	76.6	43.0	53.6	58.1	40.4	56.3	13.8	5.6
20	100.0	100.0	100.0	100.0	100.0	100.0	100.0	0.0	0.0
30	100.0	100.0	100.0	100.0	100.0	100.0	100.0	0.0	0.0
40	100.0	100.0	100.0	100.0	100.0	95.8	99.3	1.7	0.7
60	77.8	100.0	100.0	100.0	100.0	100.0	96.3	9.1	3.7
90	46.9	100.0	100.0	87.6	81.5	100.0	86.0	20.7	8.4
120	26.7	59.5	52.0	60.2	60.7	58.6	53.0	13.2	5.4
150	25.7	25.9	28.2	29.2	35.8	41.7	31.1	6.4	2.6
180	9.0	14.6	7.2	7.7	11.5	13.4	10.6	3.1	1.3

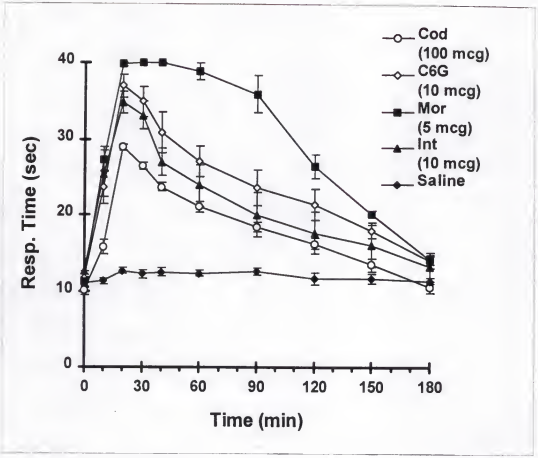
Table 3-12 : The data for the effect of intracerebroventricular administration of 5 μg of morphine on the analgesic responses (in% MPE).

Time (min)	1	2	3	4	5	6	Mean	SD	SEM
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10	100.0	11.2	31.4	77.0	25.1	36.3	46.8	34.1	13.9
20	100.0	58.4	100.0	100.0	56.1	73.6	81.4	21.3	8.7
30	100.0	45.5	86.0	100.0	53.1	65.9	75.1	23.7	9.7
40	54.3	27.6	56.6	100.0	29.9	43.6	52.0	26.4	10.8
60	14.5	29.4	41.5	100.0	27.3	28.2	40.1	30.6	12.5
90	16.4	13.6	29.1	64.3	17.7	18.7	26.6	19.2	7.8
120	1.2	-1.0	19.4	59.3	14.8	13.2	17.8	21.9	8.9
150	-3.1	-2.4	17.1	42.3	10.7	7.3	12.0	16.8	6.8
180	-6.3	-5.6	8.5	15.3	1.1	-1.5	1.9	8.5	3.5

Table 3-13 : The data for the effect of intracerebroventricular administration of the 10 μg of the intermediate on the analgesic responses (in% MPE).

Time (min)	1	2	3	4	5	6	Mean	SD	SEM
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10	-2.1	4.4	3.0	6.2	5.9	3.8	3.6	3.0	1.2
20	3.1	6.4	6.7	7.6	7.7	4.8	6.1	1.8	0.7
30	11.1	4.1	13.5	5.5	5.2	7.0	7.7	3.7	1.5
40	13.9	1.7	15.2	10.0	8.7	8.3	9.6	4.8	2.0
60	10.1	2.4	7.4	10.7	4.2	6.1	6.8	3.3	1.3
90	5.9	1.4	11.8	5.9	5.9	3.2	5.7	3.5	1.4
120	0.7	2.7	12.8	9.0	7.7	8.3	6.9	4.4	1.8
150	-0.7	4.1	6.4	3.8	7.0	8.9	4.9	3.4	1.4
180	-2.4	0.3	-1.7	0.7	6.6	6.7	1.7	4.0	1.6

Table 3-14 : The data for the effect of intracerebroventricular administration of saline on the analgesic responses (in% MPE).

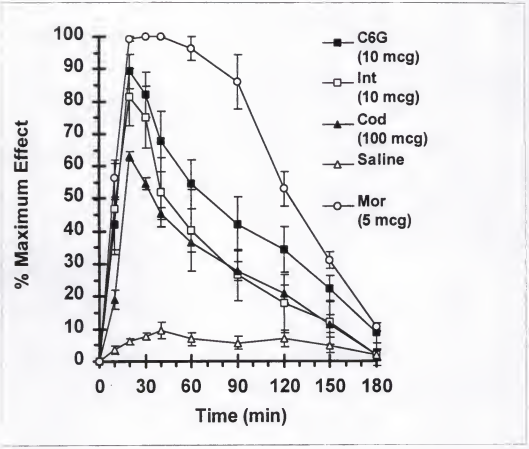


* (mcg = micrograms).

Figure 3-9 : Effect of Response Time to ICV Administration of Saline, Codeine, Intermediate, Codeine 6-Glucuronide and Morphine.

Compound	% MPE	Peak Time
Morphine (5 μg)	100 ± -	20 min
Codeine (100 μg)	63 ± 2	20 min
Codeine 6-glucuronide (10 μg)	89 ± 6	20 min
Intermediate (10 μg)	81 ± 9	20 min

Table 31-5 : % of maximum possible effect for various compounds at the peak response time after i.c.v. administration.



* (mcg = micrograms).

Figure 3-10 : Analgesic responses of rats to i.c.v. administration of saline, codeine, intermediate, codeine 6-glucuronide and morphine.

Compound	Total $AUEC_{0\text{-}3\ h}$
Morphine (5 μg)	11545 ± 744
Codeine (100 μg)	4462 ± 546
Codeine 6-glucuronide (10 μg)	8156 ± 1744
Intermediate (10 μg)	6416 ± 2602

Table 3-16 : Total $AUEC_{0\text{-}3\ h}$ values after i.c.v. administration.

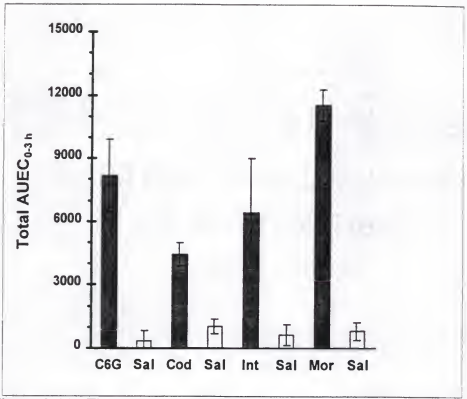


Figure 3-11 : The total area under the effect curve ($AUEC_{0-3\ h}$) after i.c.v. administration.

The area under the effect curve (AUEC) was determined from individual% MPE versus time graphs for each treatment using trapezoidal calculation (Figure 3-14) and is summarized in Table 3-26.

3.3.3 Intravenous Route

Intravenous administration resulted in significant increases in the responses associated with both codeine 6-glucuronide and the intermediate. Codeine also produced a greater response by this route compared to the subcutaneous route. As in the case of subcutaneous administration, there were differences in the peak response times.

Time (min)	1	2	3	4	5	6	Mean	SD	SEM
0	10.9	9.9	11.6	8.7	10.8	12.2	10.7	1.2	0.5
15	18.2	16.3	17.4	18.3	20.5	22.3	18.8	2.2	0.9
30	26.9	27.7	28.9	27.3	29.9	30.2	28.5	1.4	0.6
45	21.3	25.1	26.3	26.6	28.7	28.6	26.1	2.7	1.1
60	20.6	24.4	24.7	25.9	28.3	27.8	25.3	2.8	1.1
90	18.7	22.2	23.6	25.4	27.9	27.7	24.3	3.5	1.4
120	17.6	20.1	21.1	23.5	25.8	25.4	22.3	3.2	1.3
180	16.8	18.7	20.3	21.7	22.6	23.3	20.6	2.5	1.0
240	14.9	16.6	17.5	20.2	20.6	21.1	18.5	2.5	1.0
300	13.8	14.8	15.2	18.3	17.7	18.4	16.4	2.0	0.8
360	12.3	12.2	13.7	15.1	13.2	13.8	13.4	1.1	0.4

Table 3-17 : The data for the effect of subcutaneous administration of 10 mg/kg of codeine on the response time (in seconds).

Time (min)	1	2	3	4	5	6	Mean	SD	SEM
0	8.8	10.6	10.3	9.4	13.1	13.2	10.6	1.8	0.6
15	12.3	12.1	12.5	12.2	11.3	10.9	11.9	1.6	0.5
30	14.7	14.8	12.8	13.6	12.1	12.2	13.5	2.3	0.8
45	18.2	17.3	14.7	13.9	12.7	13.7	15.3	3.6	1.2
60	16.8	16.5	12.9	13.7	14.6	13.9	14.9	2.9	1.0
90	16.7	14.9	12.6	12.9	13.3	12.6	13.9	2.9	1.0
120	13.0	14.5	13.5	12.7	13.1	12.2	13.2	1.8	0.6
180	11.4	13.7	11.7	11.2	12.7	12.3	11.8	1.6	0.5
240	9.8	12.1	11.2	11.9	11.9	11.9	11.3	1.2	0.4
300	10.4	11.3	11.8	10.8	11.3	11.8	11.3	0.7	0.2
360	8.9	11.0	11.9	10.3	10.8	11.2	10.6	1.1	0.4

Table 3-18 : The data for the effect of subcutaneous administration of 10 mg/kg of codeine 6-glucuronide on the response time (in seconds).

Time (min)	1	2	3	4	5	6	Mean	SD	SEM
0	9.5	9.9	10.1	10.2	12.1	10.1	10.0	1.0	0.3
15	9.8	11.5	10.6	10.4	11.3	9.5	10.3	0.8	0.3
30	10.2	13.3	10.6	12.3	10.9	11.2	11.3	1.1	0.4
45	12.5	13.8	11.3	13.8	12.6	11.6	13.0	1.3	0.5
60	13.2	13.7	12.5	13.9	13.1	12.8	13.2	0.7	0.2
90	13.4	12.6	12.9	12.9	13.3	12.7	13.0	1.2	0.4
120	12.7	12.7	11.7	12.6	12.8	11.9	12.6	1.1	0.4
180	11.9	13.2	12.3	11.8	12.2	11.3	12.1	0.6	0.2
240	12.6	12.5	12.6	11.9	11.7	10.8	11.9	1.1	0.4
300	12.7	11.9	10.7	11.1	11.6	10.7	11.4	0.8	0.3
360	12.0	10.7	9.6	11.4	10.3	10.2	10.8	1.0	0.3

Table 3-19 : The data for the effect of subcutaneous administration of 10 mg/kg of the intermediate on the response time (in seconds).

Time (min)	1	2	3	4	5	6	MEAN	SD	SEM
0	8.1	8.5	8.6	9.8	9.6	10.8	9.8	1.4	0.5
15	9.8	10.0	8.1	10.7	9.3	9.2	9.8	0.9	0.3
30	10.1	10.8	10.2	10.2	10.0	9.9	10.3	0.5	0.2
45	11.0	10.2	11.3	10.1	10.1	11.2	10.9	0.7	0.3
60	9.8	11.1	9.8	10.0	9.9	11.7	10.6	0.8	0.3
90	8.9	10.8	9.8	10.2	10.2	12.2	10.6	1.0	0.4
120	8.6	9.3	10.7	10.3	10.3	11.9	10.3	1.0	0.4
180	10.3	8.9	11.3	9.5	10.7	11.7	10.5	0.9	0.3
240	10.6	9.4	10.7	9.7	9.8	10.6	10.1	0.5	0.2
300	11.2	8.6	11.3	9.9	9.9	10.4	9.9	1.0	0.4
360	11.1	9.7	10.6	10.1	10.4	10.2	10.2	0.6	0.2

Table 3-20 : The data for the effect of subcutaneous administration of saline on the response time (in seconds).

Time (min)	1	2	3	4	5	6	Mean	SD	SEM
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
15	25.1	21.3	20.4	30.7	33.2	36.3	27.8	6.6	2.7
30	55.0	59.1	60.9	59.4	65.4	64.7	60.8	3.9	1.6
45	35.7	50.5	51.8	57.2	61.3	59.0	52.6	9.2	3.8
60	33.3	48.2	46.1	55.0	59.9	56.1	49.8	9.6	3.9
90	26.8	40.9	42.3	53.4	58.6	55.8	46.3	12.0	4.9
120	23.0	33.9	33.5	47.3	51.4	47.5	39.4	11.0	4.5
180	20.3	29.2	30.6	41.5	40.4	39.9	33.7	8.4	3.4
240	13.7	22.3	20.8	36.7	33.6	32.0	26.5	8.9	3.6
300	10.0	16.3	12.7	30.7	23.6	22.3	19.3	7.7	3.1
360	4.8	7.6	7.4	20.4	8.2	5.8	9.0	5.7	2.3

Table 3-21 : The data for the effect of subcutaneous administration of 10 mg/kg of codeine on the analgesic responses (in% MPE).

Time (min)	1	2	3	4	5	6	Mean	SD	SEM
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
15	4.5	5.1	10.2	7.4	2.8	9.2	6.9	3.0	1.0
30	11.6	14.3	21.6	8.4	5.9	13.7	12.8	6.1	2.0
45	28.4	22.8	28.3	14.8	2.5	14.7	16.6	9.8	3.3
60	27.1	20.1	21.9	8.8	4.7	14.1	13.9	7.3	2.4
90	18.8	14.6	21.2	7.7	0.6	11.4	11.1	7.7	2.6
120	9.6	13.3	16.3	10.8	5.6	10.8	11.3	3.9	1.3
180	4.1	10.5	5.7	4.7	1.6	5.9	5.7	3.2	1.1
240	0.0	5.1	3.5	3.0	3.7	8.2	4.7	2.1	0.7
300	1.0	2.4	2.8	5.1	8.1	4.6	4.6	2.3	0.8
360	2.7	1.4	-3.5	5.4	3.1	2.9	1.9	3.3	1.1

Table 3-22 : The data for the effect of subcutaneous administration of 10 mg/kg of the Intermediate on the analgesic responses (in% MPE).

Time (min)	1	2	3	4	5	6	Mean	SD	SEM
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
15	0.3	1.0	5.3	1.7	3.8	0.7	1.6	2.8	1.0
30	3.3	2.3	11.3	1.7	8.9	7.0	4.5	5.7	2.0
45	19.0	9.8	13.0	4.0	14.7	12.1	11.1	4.2	1.5
60	15.7	12.1	12.6	8.0	11.2	12.4	10.0	3.6	1.3
90	18.6	12.8	9.0	9.4	7.0	9.1	8.6	2.8	1.0
120	18.0	10.5	9.3	5.4	7.7	8.1	7.2	2.9	1.0
180	10.5	7.9	11.0	7.4	9.3	5.4	6.9	3.7	1.3
240	11.4	10.2	8.6	8.4	3.5	5.7	5.8	4.3	1.5
300	8.8	10.5	6.6	2.0	5.1	3.0	4.2	4.2	1.5
360	9.5	8.2	2.7	-1.7	4.8	4.0	1.9	5.2	1.8

Table 3-23 : The data for the effect of subcutaneous administration of 10 mg/kg of codeine 6-glucuronide on the analgesic responses (in% MPE).

Time (min)	1	2	3	4	5	6	Mean	SD	SEM
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
15	11.3	11.6	13.1	13.9	8.3	0.7	8.0	6.5	2.5
30	15.6	17.5	16.9	20.8	16.2	-1.1	11.8	9.7	3.7
45	-2.8	13.4	2.1	20.2	17.8	-7.1	6.4	10.7	4.0
60	-1.1	14.4	7.6	21.5	15.0	-4.9	7.7	9.7	3.7
90	-4.3	9.1	3.8	18.6	12.7	-0.4	5.5	8.3	3.1
120	-1.1	7.2	10.3	17.0	13.1	-2.8	6.2	7.7	2.9
180	-5.0	11.6	-3.1	17.7	12.4	-1.8	4.7	9.0	3.4
240	-1.4	10.0	5.5	15.8	10.5	-3.2	5.1	7.4	2.8
300	-6.7	5.9	2.1	18.3	10.5	-7.4	2.8	9.5	3.6
360	-5.0	3.8	-7.9	12.6	9.9	-1.8	1.0	7.9	3.0

Table 3-24 : The data for the effect of subcutaneous administration of saline on the analgesic responses (in% MPE).

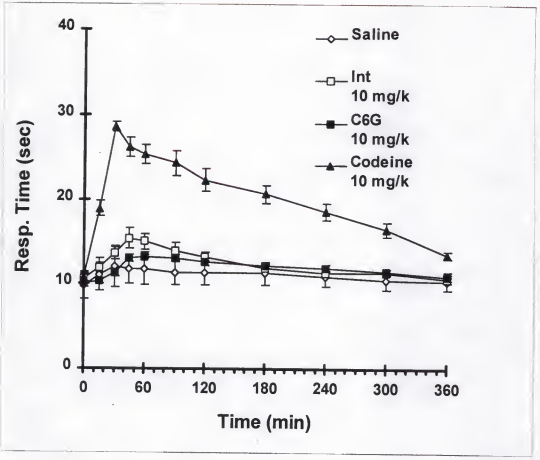


Figure 3-12 : Effect of response time to s.q. administration of saline, codeine, intermediate and codeine 6-glucuronide.

Compound	% MPE	Peak Time
Codeine (10 mg/kg)	61 ± 5	30 min
Codeine 6-glucuronide (10 mg/kg)	11 ± 2	45 min
Intermediate (10 mg/kg)	17 ± 3	45 min

Table 3-25 : % of maximum possible effect for various compounds at the peak response time after s.q. administration.

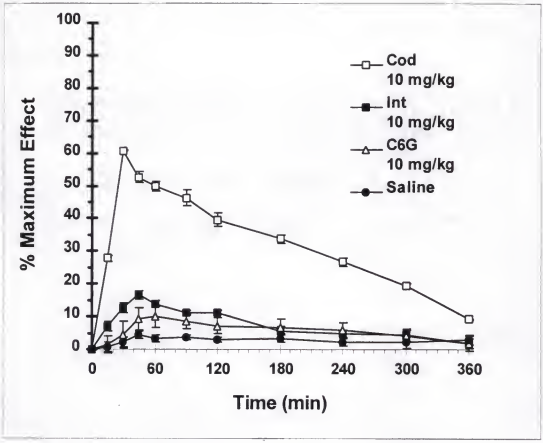


Figure 3-13 : Analgesic responses to s.q. administration of saline, codeine, intermediate and codeine 6-glucuronide.

Compound	Total $AUEC_{0\text{-}6\ h}$
Codeine (10 mg/kg)	10886 ± 646
Codeine 6-glucuronide (10 mg/kg)	1765 ± 159
Intermediate (10 mg/kg)	2321 ± 269

Table 3-26 : Total $AUEC_{0\text{-}6\ h}$ values after s.q. administration.

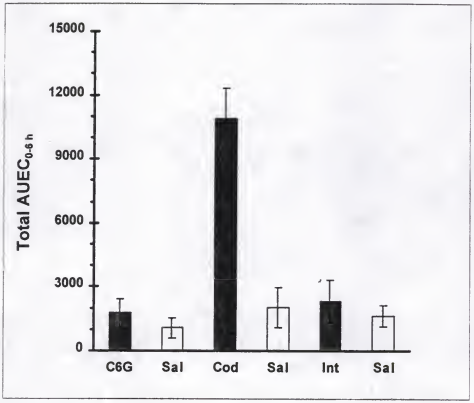


Figure 3-14 : The total area under the effect curve ($AUEC_{0\text{-}6\ h}$) after s.q. administration.

The response time (Tables 3-27 to 3-30) for all the treatments was transformed into% of maximum effect data (Tables 3-31 to 3-34). The average values from the data sets were then plotted as a function of time (Figures 3-15 and 3-16). Codeine exhibited a significant response, that is, 98 $\pm$ 4% of the MPE. Codeine 6-glucuronide and the intermediate produced responses which were 55 $\pm$ 3 and 66 $\pm$ 3% of MPE, respectively. The% MPE at peak response time for all the compounds is summarized in Table 3-35. The area under the effect curve (AUEC) was determined from individual% MPE versus time graphs for each treatment using trapezoidal calculation (Figure 3-17) and is summarized in Table 3-36.

Time (min)	1	2	3	4	5	6	Mean	SD	SEM
0	8.7	11.8	9.8	12.8	10.1	11.4	11.1	1.6	0.6
10	27.6	31.3	33.7	31.7	29.8	27.7	29.3	3.4	1.3
20	38.4	37.9	36.9	40.0	39.1	37.2	38.5	1.2	0.5
30	35.9	34.1	33.6	37.7	36.3	34.5	35.7	3.1	1.2
40	32.7	33.8	32.9	34.3	35.4	32.8	33.3	3.6	1.4
60	23.8	22.2	26.9	26.4	30.6	25.1	25.7	2.7	1.0
90	19.7	18.1	22.7	23.1	24.3	23.7	21.7	2.3	0.9
120	18.1	17.7	20.3	20.3	21.0	18.8	19.0	1.6	0.6
180	14.8	15.2	19.7	19.7	19.9	16.3	17.2	2.5	0.9
240	13.2	14.4	16.8	16.6	9.4	13.2	13.8	2.5	0.9
300	11.6	12.3	14.7	14.5	10.0	12.7	12.6	1.6	0.6

Table 3-27 : The data for the effect of intravenous administration of 10 mg/kg of codeine on the response time (in seconds).

Time (min)	1	2	3	4	5	6	Mean	SD	SEM
0	12.1	11.7	10.6	12.6	10.3	11.6	11.7	0.9	0.4
10	19.9	17	19.7	21.2	14.4	13.9	17.0	3.3	1.3
20	26.6	25.8	26.9	25.7	23.9	22.1	24.5	2.5	0.9
30	28.2	29.6	28.3	29.5	26.4	25.3	27.3	2.2	0.8
40	26.7	27.7	26.2	22.3	24.1	23.2	24.5	2.5	0.9
60	19.4	22.3	24.7	19.9	22.9	20.8	20.9	2.7	1.0
90	16.3	18.8	22.3	17.8	17.7	17.8	18.1	2.1	0.8
120	14.7	16.9	20.9	15.6	15.5	16.9	16.4	2.2	0.8
180	13.9	14.6	19.2	14.4	14.1	15.4	15.1	1.9	0.7
240	12.6	11.8	18.1	13.3	12.8	14.8	13.8	2.1	0.8
300	11.8	10.9	16.3	12.8	10.9	13.9	12.8	1.9	0.7

Table 3-28 : The data for the effect of intravenous administration of 10 mg/kg of codeine 6-glucuronide on the response time (in seconds).

Time (min)	1	2	3	4	5	6	Mean	SD	SEM
0	10.1	11.6	9.1	12.2	11.2	13	11.3	1.3	0.5
10	17.6	20.5	15.7	19.1	17.5	18.2	17.9	1.6	0.6
20	23.9	24.8	24.2	24.5	23.6	25.1	24.4	0.5	0.2
30	32.7	33.9	30.7	29.1	28.7	29.6	30.4	2.2	0.8
40	27.9	26.5	25.1	25.2	26.0	27.7	25.8	2.0	0.8
60	25.4	19.0	23.2	23.9	22.3	22.3	22.4	2.1	0.8
90	22.4	17.6	18.1	21.2	20.8	21.1	20.0	1.8	0.7
120	18.8	16.5	16.4	18.4	17.7	19.7	18.0	1.2	0.5
180	14	15.6	14.8	17.1	17.3	17.8	16.3	1.5	0.6
240	13.7	14.8	12.7	16.2	16.6	16.9	15.1	1.6	0.6
300	12.8	13.1	11.1	15.5	14.7	14.6	13.5	1.5	0.6

Table 3-29 : The data for the effect of intravenous administration of 10 mg/kg of the intermediate on the response time (in seconds).

Time (min)	1	2	3	4	5	6	Mean	SD	SEM
0	9.3	9.9	11.1	10.1	13.0	12.2	11.2	1.5	0.6
10	10.8	10.3	10.7	10.4	12.7	12.8	11.5	1.2	0.5
20	10.6	10.6	11.0	11.1	11.8	13.1	11.6	1.0	0.4
30	11.3	10.8	11.9	10.8	13.5	12.7	12.0	1.1	0.4
40	11.0	11.1	12.3	10.7	12.2	12.2	11.9	1.0	0.4
60	10.9	11.2	11.6	11.1	13.3	12.8	12.1	1.2	0.5
90	11.3	11.2	11.7	10.0	12.7	12.9	11.8	1.1	0.4
120	11.6	10.8	12.2	11.5	12.2	12.3	11.9	0.7	0.3
180	10.9	10.9	11.9	11.3	13	12.6	11.9	0.9	0.3
240	11.1	10.6	11.3	10.6	13.1	13.6	11.9	1.3	0.5
300	10.6	10.9	11.9	9.8	13.2	13.2	11.7	1.3	0.5

Table 3-30 : The data for the effect of intravenous administration of saline on the response time (in seconds).

Time (min)	1	2	3	4	5	6	MEAN	SD	SEM
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10	60.4	69.1	79.1	69.5	65.9	57.0	66.8	7.1	2.9
20	97.9	99.6	89.7	100.0	97.0	94.2	98.3	13.3	4.2
30	86.9	79.1	78.8	91.5	87.6	80.8	84.1	4.8	2.0
40	76.7	78.0	76.5	79.0	84.6	74.8	78.3	3.1	1.3
60	48.2	36.9	56.6	50.0	68.6	47.9	51.4	9.6	3.9
90	35.1	22.3	42.7	37.9	47.5	43.0	36.6	9.0	3.4
120	30.0	20.9	34.8	27.6	36.5	25.9	27.1	7.7	2.9
180	19.5	12.1	32.8	25.4	32.8	17.1	20.9	10.0	3.8
240	14.4	9.2	23.2	14.0	-2.3	6.3	9.5	8.6	3.3
300	9.3	1.8	16.2	6.3	-0.3	4.5	5.0	6.4	2.4

Table 3-31 : The data for the effect of intravenous administration of 10 mg/kg of codeine on the analgesic responses (in% MPE).

Time (min)	1	2	3	4	5	6	Mean	SD	SEM
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10	28.0	18.7	31.0	31.4	13.8	8.1	18.8	12.0	4.5
20	52.0	49.8	55.4	47.8	45.8	37.0	45.0	9.8	3.7
30	57.7	63.3	60.2	61.7	54.2	48.2	55.2	8.1	3.1
40	52.3	56.5	53.1	35.4	46.5	40.8	45.0	9.8	3.7
60	26.2	37.5	48.0	26.6	42.4	32.4	32.4	11.4	4.3
90	15.1	25.1	39.8	19.0	24.9	21.8	22.4	9.2	3.5
120	9.3	18.4	35.0	10.9	17.5	18.7	16.5	9.6	3.6
180	6.5	10.2	29.3	6.6	12.8	13.4	11.8	8.4	3.2
240	1.8	0.4	25.5	2.6	8.4	11.3	7.4	8.9	3.4
300	-1.1	-2.8	19.4	0.7	2.0	8.1	3.9	7.6	2.9

Table 3-32 : The data for the effect of intravenous administration of 10 mg/kg of codeine 6-glucuronide on the analgesic responses (in% MPE).

Time (min)	1	2	3	4	5	6	Mean	SD	SEM
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10	25.1	31.3	21.4	24.8	21.9	19.3	22.8	4.9	1.8
20	46.2	46.5	48.9	44.2	43.1	44.8	45.5	1.9	0.7
30	75.6	78.5	69.9	60.8	60.8	61.5	66.2	8.5	3.2
40	59.5	52.5	51.8	46.8	51.4	54.4	50.2	7.7	2.9
60	51.2	26.1	45.6	42.1	38.5	34.4	38.3	8.8	3.3
90	41.1	21.1	29.1	32.4	33.3	30.0	30.3	6.4	2.4
120	29.1	17.3	23.6	22.3	22.6	24.8	23.1	3.5	1.3
180	13.0	14.1	18.4	17.6	21.2	17.8	17.5	3.0	1.1
240	12.0	11.3	11.7	14.4	18.8	14.4	13.2	3.0	1.1
300	9.0	5.3	6.5	11.9	12.2	5.9	7.6	3.6	1.3

Table 3-33 : The data for the effect of intravenous administration of 10 mg/kg of the intermediate on the analgesic responses (in % MPE).

Time (min)	1	2	3	4	5	6	Mean	SD	SEM
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10	4.9	1.3	-1.4	1.0	-1.1	2.2	0.9	2.2	0.8
20	4.2	2.3	-0.3	3.3	-4.4	3.2	1.0	3.1	1.2
30	6.5	3.0	2.8	2.3	1.9	1.8	2.8	1.8	0.7
40	5.5	4.0	4.2	2.0	-3.0	0.0	2.1	2.9	1.1
60	5.2	4.3	1.7	3.3	1.1	2.2	3.1	1.5	0.6
90	6.5	4.3	2.1	-0.3	-1.1	2.5	2.1	2.7	1.0
120	7.5	3.0	3.8	4.7	-3.0	0.4	2.3	3.5	1.3
180	5.2	3.3	2.8	4.0	0.0	1.4	2.2	2.3	0.9
240	5.9	2.3	0.7	1.7	0.4	5.0	2.2	2.4	0.9
300	4.2	3.3	2.8	-1.0	0.7	3.6	1.5	2.8	1.1

Table 3-34 : The data for the effect of intravenous administration of saline on the analgesic responses (in% MPE).

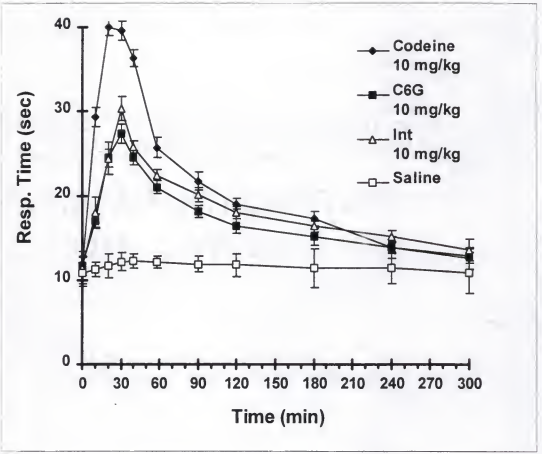


Figure 3-15 : Effect of response time to i.v. administration of saline, codeine, intermediate and codeine 6-glucuronide.

Compound	% MPE	Peak Time
Codeine (10 mg/kg)	98 ± 4	20 min
Codeine 6-glucuronide (10 mg/kg)	55 ± 3	30 min
Intermediate (10 mg/kg)	66 ± 3	30 min

Table 3-35 : % of maximum possible effect for various compounds at the peak response time after i.v. administration.

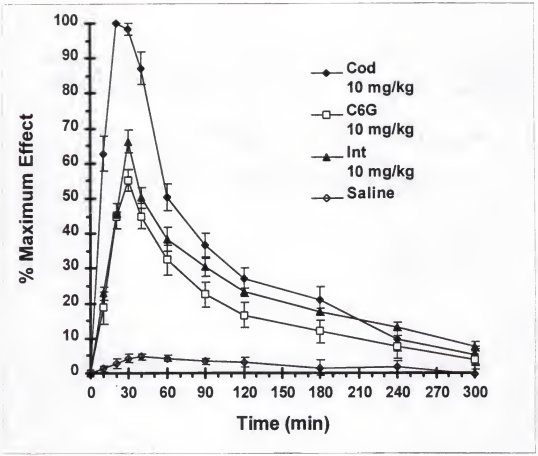


Figure 3-16 : Analgesic Responses to i.v. Administration of Saline, Codeine, Intermediate and Codeine 6-Glucuronide.

Compound	Total $AUEC_{0\text{-}5\ h}$
Codeine (10 mg/kg)	8718 ± 960
Codeine 6-glucuronide (10 mg/kg)	4781 ± 589
Intermediate (10 mg/kg)	5933 ± 289

Table 3-36 : Total $AUEC_{0\text{-}5\ h}$ values after i.v. administration.

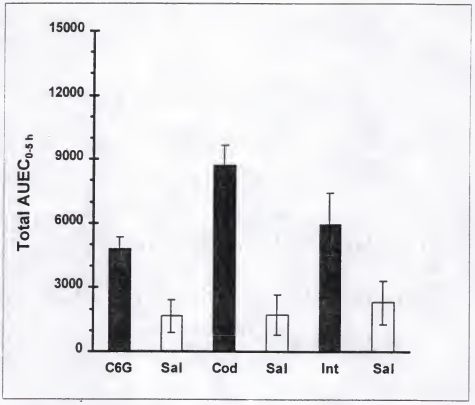


Figure 3-17 : The total area under the effect curve ($AUEC_{0\text{-}5\ h}$) after i.v. administration.

3.4 Immune Studies

Codeine and its 6-glucuronide demonstrated inhibition of proliferation in the PHA-stimulated lymphocytes, with maximal differences in cell inhibition of the parent compound and its metabolite in the lower, physiologically relevant concentration range of 0.156-5 μg/ml, as seen in Figure 3-18. Similarly, in the case of the PMA-stimulated lymphocytes, there was very little suppression observed at lower concentrations compared to the higher concentrations (Figure 3-19). Codeine and codeine 6-glucuronide also inhibited the MLR-stimulated lymphocytes in a concentration-dependent fashion (Figure 3-20).

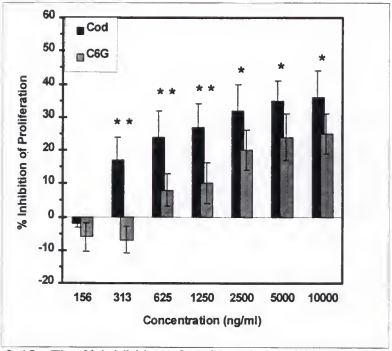


Figure 3-18 : The % inhibition of proliferation of PHA-stimulated T lymphocytes after the addition of codeine and codeine 6-glucuronide. * and * * indicate a p-value of 0.05 and 0.01, respectively.

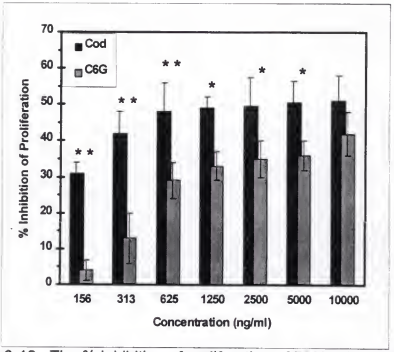


Figure 3-19 : The % inhibition of proliferation of PMA-stimulated T lymphocytes after the addition of codeine and codeine 6-glucuronide. * and * * indicate a p-value of 0.05 and 0.01, respectively.

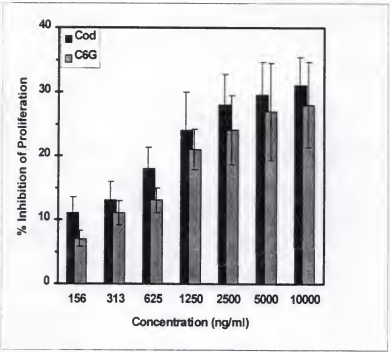


Figure 3-20 : The% inhibition of proliferation of MLR-stimulated T lymphocytes after the addition of codeine and codeine 6-glucuronide.

lymphocytes after the addition of codeine and codeine 6-glucuronide.Similar to the effects of codeine and codeine 6-glucuronide, morphine and its 6-glucuronide exhibited greatest inhibition of proliferation in the PHA-stimulated cells (Figure 3-21). In the case of PMA-stimulated lymphocytes (Figure 3-22), morphine and morphine 6-glucuronide demonstrated almost negligible inhibition at lower concentrations (<1.25 μg/ml). These compounds also showed inhibition of the Mixed Lymphocyte Reaction (Figure 3-23).

When the intermediate was tested against codeine 6-glucuronide, it showed a greater inhibition over the entire concentration range tested (0.156 to 10 μg/ml) in all of the assays, that is, PHA (Figure 3-24), PMA (Figure 3-25) and the MLR (Figure 3-26) models.

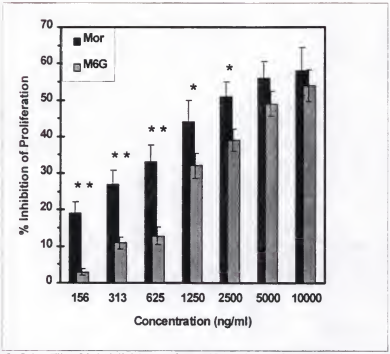


Figure 3-21 : The% inhibition of proliferation of PHA-stimulated T lymphocytes after the addition of morphine and morphine 6-glucuronide. * and * * indicate a p-value of 0.05 and 0.01, respectively.

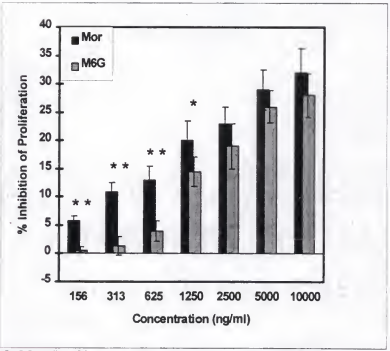


Figure 3-22 : The% inhibition of proliferation of PMA-stimulated T lymphocytes after the addition of morphine and morphine 6-glucuronide. * and * * indicate a p-value of 0.05 and 0.01, respectively.

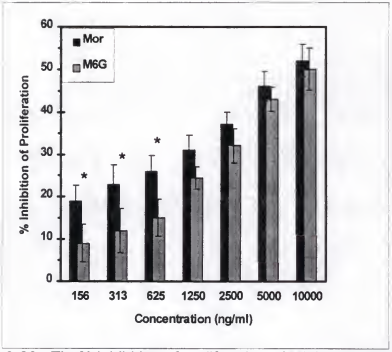


Figure 3-23 : The% inhibition of proliferation of MLR-stimulated T lymphocytes after the addition of morphine and morphine 6-glucuronide. * and * * indicate a p-value of 0.05 and 0.01, respectively.

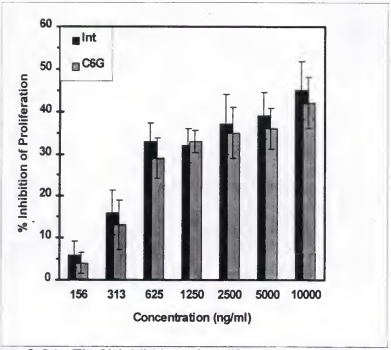


Figure 3-24 : The% inhibition of proliferation of PHA-stimulated T lymphocytes after the addition of codeine 6-glucuronide and the intermediate.

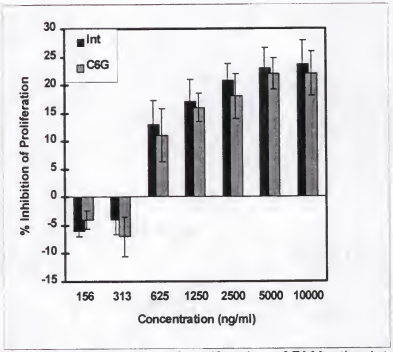


Figure 3-25 : The% inhibition of proliferation of PMA-stimulated T lymphocytes after the addition of codeine 6-glucuronide and the intermediate.

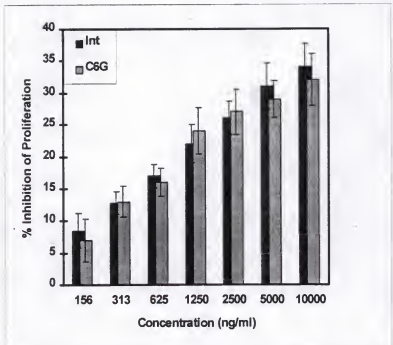


Figure 3-26 : The% inhibition of proliferation of MLR-stimulated T lymphocytes after the addition of codeine 6-glucuronide and the intermediate.

3.5 Receptor Binding Studies

Morphine exhibited the highest affinity in the receptor binding assays, with an IC_{50} value of 1.8 $\pm$ 0.6 nM (Table 3-9). In the case of codeine, however, the binding curve was shifted significantly to the right of the concentration range (IC_{50} = 1.5 $\pm$ 0.4 μM) indicating a much weaker affinity for the μ receptor. Codeine 6-glucuronide had a slightly lower binding compared to codeine (IC_{50} = 4.4 $\pm$ 0.2 μM). The intermediate, on the other hand, exhibited a slightly higher μ receptor binding affinity compared to codeine and codeine 6-glucuronide (IC_{50} = 0.13 $\pm$ 0.06 μM).

The raw counts per minute (CPM) data of each compound were converted into the more standardized form, that is, % of total binding. The receptor binding profiles of morphine (Figures 3-27 and 3-28), codeine (Figures 3-29 and 3-30), codeine 6-glucuronide (Figures 3-31 and 3-32) and the intermediate (Figures 3-33 and 3-34) are represented in the graphs below.

Parameters	Mor	Cod	C6G	Int
T (cpm)	3358 $\pm$ 343	2609 $\pm$ 350	2249 $\pm$ 107	2479 $\pm$ 481
NS (cpm)	961 $\pm$ 97	876 $\pm$ 24	706 $\pm$ 64	779 $\pm$ 141
N	0.98 $\pm$ 0.42	0.96 $\pm$ 0.26	1.02 $\pm$ 0.28	1.13 $\pm$ 0.36
IC_{50}	1.81 $\pm$ 0.58 nM	1.49 $\pm$ 0.39 μM	4.4 $\pm$ 0.23 μM	0.13 $\pm$ 0.06 μM

Table 3-37 : The μ receptor binding parameters for morphine, codeine, codeine 6-glucuronide and the intermediate.

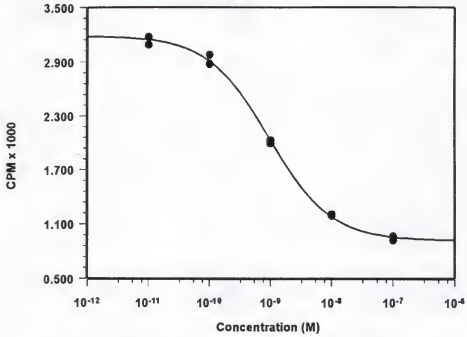


Figure 3-27 : The receptor binding profile of morphine. (CPM vs concentration).

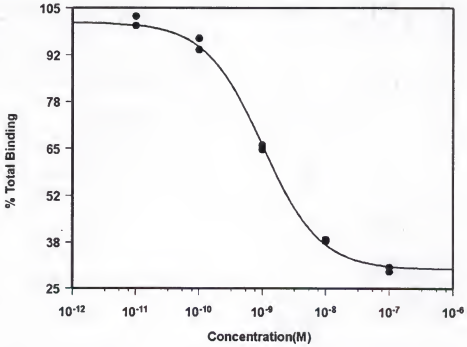


Figure 3-28 : The receptor binding profile of morphine. (% total binding vs concentration).

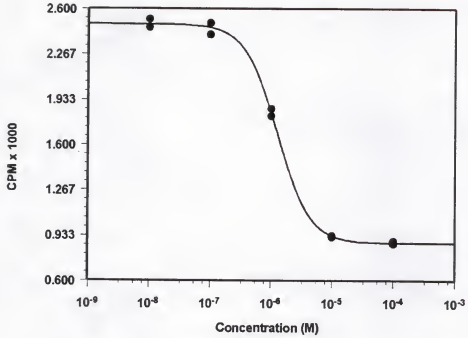


Figure 3-29 : The receptor binding profile of codeine. (CPM vs concentration).

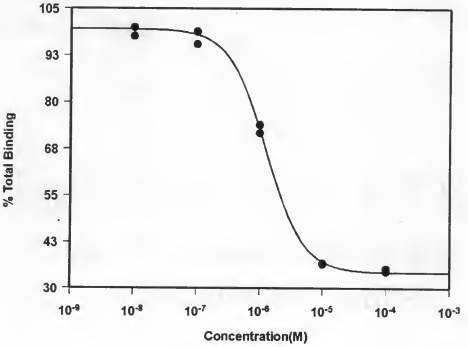


Figure 3-30 : The receptor binding profile of codeine. (% total binding vs concentration).

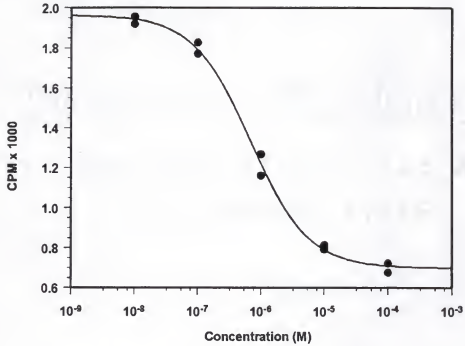


Figure 3-31 : The receptor binding profile of codeine 6-glucuronide. (CPM vs concentration).

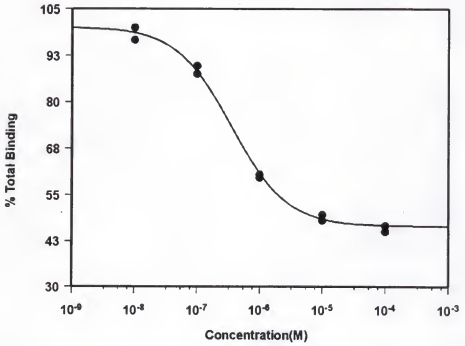


Figure 3-32 : The receptor binding profile of codeine 6-glucuronide. (% total binding vs concentration).

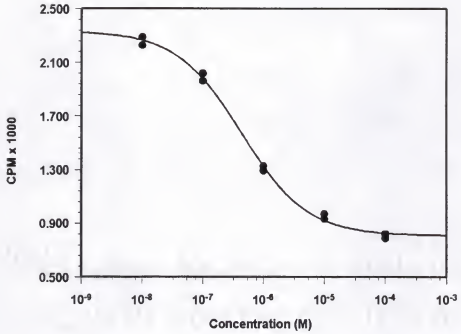


Figure 3-33 : The receptor binding profile of the intermediate. (CPM vs concentration).

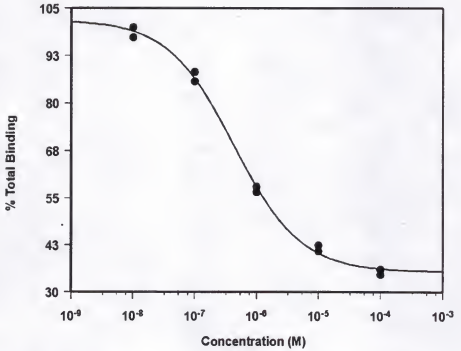


Figure 3-34 : The receptor binding profile of the intermediate. (% total binding vs concentration).

3.6 Plasma and Brain Concentrations

System 2 was used to determine concentrations of codeine, morphine and their glucuronides in rat plasma and brain samples. Extractions were done using disposable Clean Screen® columns as in the case of blank human urine used to develop the HPLC method. However, for the rat plasma and brain samples, a 10% ammonium hydroxide solution in methanol was required to obtain good recoveries of the polar glucuronide metabolites. The recoveries were better than 80% for all the compounds.

Intracerebroventricular administration of codeine (Figure 3-35) in rats resulted in the partial conversion of codeine to morphine in the brain (Table 3-38). However, no drug was detected in the plasma. When codeine 6-glucuronide was administered directly into the brain, a small amount of morphine 6-glucuronide was detected in the brain (Table 3-39) but no drug was detected in the plasma.

After subcutaneous administration of codeine, it was seen that some codeine was converted into morphine and morphine 3-glucuronide in the plasma. Neither morphine 6-glucuronide or codeine 6-glucuronide were detected in the plasma. However, both morphine 6-glucuronide and codeine 6-glucuronide were present in small quantities in the brain of some of the rats. After subcutaneous administration of codeine 6-glucuronide, no drug or its metabolites was detected either in the plasma or the brain. Therefore, this route of administration was not pursued.

After intravenous administration of codeine (Figure 3-36), some of the codeine was converted into morphine, morphine 3-glucuronide and codeine 6-glucuronide were found in the plasma (Table 3-40). In the brain, small amounts

of both morphine 6-glucuronide and codeine 6-glucuronide were detected (Table 3-41). Codeine 6-glucuronide administration resulted in the presence of only codeine 6-glucuronide in the plasma (Table 3-42). However, in the brain, small amounts of both codeine 6-glucuronide and morphine 6-glucuronide were detected (Table 3-43). The results obtained after administration of codeine and codeine 6-glucuronide by the various routes are summarized in Table 3-44.

Sample number	Codeine (ng/g)	Morphine (ng/g)
1	9652	1803
2	7965	1046
3	8242	1287
4	7191	1311
5	6647	1207
6	8018	1379
Mean ± SD	**7953 ± 1026**	**1339 ± 254**

Table 3-38 : Concentrations in the rat brain after i.c.v. administration of 100 μg codeine.

Sample number	C6G (ng/g)	M6G (ng/g)
1	815	63
2	744	43
3	917	57
4	1033	78
5	668	53
6	791	69
Mean + SD	**828 ± 130**	**61 ± 12**

Table 3-39 : Concentrations in the rat brain after i.c.v. administration of 10 μg codeine 6-glucuronide.

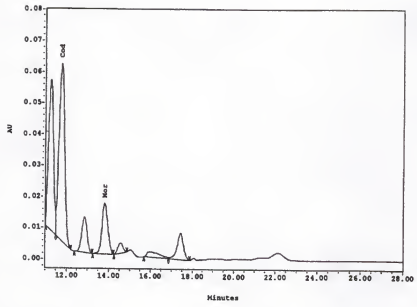


Figure 3-35 : Chromatogram of a brain sample after administration of 100 μg of codeine i.c.v. in the rat.

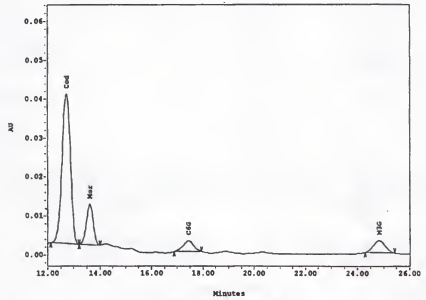


Figure 3-36 : Chromatogram of a plasma sample after administration of 10 mg/kg codeine i.v. in the rat.

Sample number	Codeine (ng/ml)	Morphine (ng/ml)	C6G (ng/ml)	M3G (ng/ml)
1	2187	199	28	469
2	3352	239	36	564
3	2678	178	ND	447
4	1787	113	ND	239
5	1886	146	ND	348
6	2013	167	21	457
Mean ± SD	**2313 ± 596**	**174 ± 43**	**28 ± 8**	**421 ± 112**

Table 3-40 : Concentrations in the rat plasma after i.v. administration of 10 mg/kg of codeine.

Sample number	Codeine (ng/g)	Morphine (ng/g)	C6G (ng/g)	M6G (ng/g)
1	247	51	27	21
2	319	49	34	25
3	276	42	23	ND
4	231	37	21	ND
5	198	29	ND	ND
6	257	45	ND	18
Mean ± SD	**255 ± 41**	**42 ± 8**	**26 ± 6**	**21 ± 4**

Table 3-41 : Concentrations in the rat brain after i.v. administration of 10 mg/kg of codeine.

Sample number	C6G (ng/ml)
1	4736
2	3773
3	4062
4	5168
5	4183
6	3137
Mean ± SD	**4177 ± 715**

Table 3-42 : Concentrations in the rat plasma after i.v. administration of 10 mg/kg codeine 6-glucuronide.

Sample number	C6G (ng/g)	M6G (ng/g)
1	197	29
2	161	ND
3	258	27
4	396	41
5	142	ND
6	218	35
Mean ± SD	**229 ± 92**	**33 ± 6**

Table 3-43 : Concentrations in the rat brain after i.v. administration of 10 mg/kg codeine 6-glucuronide.

Drug Administration	Plasma	Brain
Codeine - ICV	-	C, M
C6G - ICV	-	C6G, M6G
Codeine - SQ	C, M, M3G	C, M, C6G, M6G
C6G - SQ	-	-
Codeine - IV	C, M, C6G, M3G	C, M, C6G, M6G
C6G - IV	C6G	C6G, M6G

Table 3-44 : Qualitative summary of results after administration of codeine and codeine 6-glucuronide by various routes.

CHAPTER 4
DISCUSSION

The overall aim of this research project was to examine and compare the analgesic and immunomodulatory effects of codeine and codeine 6-glucuronide. To achieve this objective the following hypotheses were formulated.

1. Codeine 6-glucuronide, like morphine 6-glucuronide, possesses analgesic activity.
2. The glucuronide metabolites of codeine and morphine are less immunosuppressive than their parent compounds.

The rat was found to be a good model for the analgesia studies since negligible amounts of codeine 6-glucuronide are present in the rat plasma and urine after codeine administration. This allowed direct comparison of the antinociceptive activities of codeine 6-glucuronide with codeine without the contribution of codeine 6-glucuronide to the analgesia of codeine.

An HPLC-UV based method was successfully developed for the analysis of codeine, morphine, codeine 6-glucuronide, morphine 6-glucuronide and morphine 3- glucuronide in biological samples, that is, human urine, rat plasma and rat brain. An efficient solid phase extraction method was used to obtain clean extracts and excellent recoveries in excess of 80% for all the compounds of interest. Calibration curves for each drug were linear over the concentration range 0-500 ng/ml in all the biological samples with a correlation coefficient of 0.99 or better.

During the synthetic procedure, an intermediate compound formed after the first reaction step was isolated and characterized. This intermediate represents the glucuronic acid moiety attached to the 6-position of codeine with intact acetyl/methyl groups. It appears to be comparatively more lipophilic than codeine 6-glucuronide and should be capable of crossing the BBB more easily. In these studies the exhibited antinociceptive activity which was similar to that of codeine 6-glucuronide itself. This indicates that this kind of "prodrug" might be a viable approach for delivering the potent glucuronides across the BBB.

Intracerebroventricular administrations of morphine, codeine, codeine 6-glucuronide and the intermediate resulted in significant analgesic responses. All the compounds exhibited greater areas under the effect curve (AUECs) compared to saline treatment ($p<0.01$). Morphine (dose = 5 μg) AUEC value was seen to be statistically higher than codeine 6-glucuronide (dose = 10 μg), intermediate (dose = 10 μg) and codeine (dose = 100 μg), with $p< 0.01$. Both codeine 6-glucuronide and the intermediate exhibited larger AUECs compared to codeine ($p<0.05$). However, there were no statistically significant differences between codeine 6-glucuronide and the intermediate. Using this method, the rank order of effectiveness based on the AUEC values was morphine > codeine 6-glucuronide $\simeq$ intermediate > codeine > saline.

The analgesic effects of codeine 6-glucuronide and the intermediate were not statistically different from saline treatment after the subcutaneous studies. This lack of activity is probably associated with the limited ability of the polar glucuronides to cross the BBB and the poor absorption of these hydrophilic molecules from the subcutaneous tissue into the systemic circulation. Codeine (dose = 10 mg/kg) exhibited a statistically significant AUEC ($p<0.01$) compared

to codeine 6-glucuronide (dose = 10 mg/kg), intermediate (dose = 10 mg/kg) and saline treatments. There were no significant differences among the other treatments, that is, codeine 6-glucuronide, intermediate and saline. Comparison of total AUEC values indicated that at the dose administered, the order of effectiveness was codeine >> codeine 6 -glucuronide $\simeq$ intermediate $\simeq$ saline.

The intravenous route was chosen in order to remove one of the rate-limiting factors, that is, absorption. There was a significant increase in the responses associated with both codeine 6-glucuronide (dose = 10 mg/kg) and the intermediate (dose = 10 mg/kg). Codeine (dose = 10 mg/kg) also produced a greater response by this route compared to the subcutaneous route. This indicates that absorption of these compounds from the subcutaneous tissue can represent a significant barrier to the pharmacodynamic responses. All of the compounds exhibited greater areas under the effect curve compared to saline treatment ($p<0.01$). The codeine AUEC value was higher than codeine 6-glucuronide and the intermediate ($p<0.01$). However, there were no significant differences between the AUECs of codeine 6-glucuronide and the intermediate. Comparison of total AUEC values indicate that at the dose administered, the order of effectiveness was codeine > codeine 6 -glucuronide $\simeq$ intermediate> saline.

All four agents tested, that is, codeine, morphine, morphine 6-glucuronide and codeine 6-glucuronide, demonstrated an immunomodulatory effect in all of the three different *in vitro* systems. Cell viability was greater than 98% in all cases as determined by the tryphan blue staining method. The differences between the effects of codeine and its 6-glucuronide in the PHA assay were more apparent at lower concentrations, that is, 0.156 to 0.625 μg/ml ($p<0.01$).

This data suggests that both codeine and codeine 6-glucuronide inhibit the T cell responses via the first signal activation pathway, which is mediated through interleukin-2 and calcium channel pathways.

In the case of PMA-stimulated lymphocytes, the 6-glucuronide showed no inhibition in the lower, physiologically relevant concentrations (0.156 and 0.313 μg/ml) compared to codeine. The 6-glucuronide demonstrated significantly less inhibition than codeine in all concentrations tested ($p < 0.05$). Codeine and codeine 6-glucuronide also inhibited the MLR-stimulated lymphocytes in a dose-dependent fashion. However, there was no statistical difference between the effects of the two agents.

Morphine and its 6-glucuronide also inhibited the PHA-stimulated cells in a concentration-dependent manner. Morphine 6-glucuronide, like codeine 6-glucuronide, produced almost no inhibition in the lower concentration range. In the concentration range of 0.156-2.5 μg/ml, the inhibitory effects of the glucuronide metabolite were less compared to its parent compound ($p<0.05$). As in the case of codeine and codeine 6-glucuronide, the differences were more apparent in the lower concentrations, that is, 0.156 to 0.625 μg/ml ($p<0.01$).

In the case of PMA-stimulated lymphocytes, morphine and morphine 6-glucuronide demonstrated almost negligible inhibition at lower concentrations (<1.25 μg/ml).. At higher concentrations, however, both compounds showed a marked inhibition of proliferation. These compounds also showed inhibition of the mixed lymphocyte reaction (MLR). The 6-glucuronide of morphine showed less immunosuppression compared to morphine over the entire concentration range (0.156-10 μg/ml). The decreased immunosuppressive effects of morphine 6-

glucuronide in comparison to morphine were statistically significant at concentrations below 0.625 μg/ml ($p<0.05$).

Both morphine and codeine exert their effects by interacting with the μ opioid receptors in the central nervous system. The stronger analgesic activity of morphine compared to codeine is usually attributed to its high receptor binding affinity. In this study, the affinity profiles of morphine, codeine, codeine 6-glucuronide and the intermediate towards the μ opioid receptor were examined. It was seen that morphine exhibited the highest affinity, with an IC_{50} value of 1.8 ± 0.6. In the case of codeine, however, the binding curve was shifted significantly to the right of the concentration range (IC_{50} = 1.5 ± 0.4 μM), indicating a much weaker affinity for the μ receptor. This is in agreement with reports in the literature, which suggest a 1000 to 3000 fold less affinity for codeine compared to morphine (Chen et al., 1990 ; Mignat et al., 1995). Codeine 6-glucuronide exhibited slightly lower binding compared to codeine (IC_{50} = 4.4 ± 0.2 μM). This indicated that glucuronidation of codeine did not change the receptor affinity significantly. The intermediate, on the other hand, exhibited a higher μ receptor binding compared to codeine and codeine 6-glucuronide (IC_{50} = 0.13 ± 0.06 μM). This enhanced binding might be due to the fact that the intermediate is more lipophilic in nature than codeine and codeine 6-glucuronide.

After intracerebroventricular administration of codeine, some of the codeine was converted into morphine in the brain. No codeine 6-glucuronide or any morphine glucuronides were detected. There was no drug detected in the plasma. When codeine 6-glucuronide was administered, there was a partial formation of morphine 6-glucuronide from codeine 6-glucuronide. As in the case of codeine administration, no drug was detected in the plasma.

After subcutaneous administration of codeine, it was seen that some of the codeine was converted into morphine in the plasma. Morphine 3-glucuronide was also detected in the plasma, but no morphine 6-glucuronide or codeine 6-glucuronide were detected. In the brain, however, both morphine 6-glucuronide and codeine 6-glucuronide were detected. This indicates that the glucuronides can cross from the blood to the brain. In the case of subcutaneous administration of codeine 6-glucuronide, however, no drugs could be detected either in the plasma or the brain. This is most likely due to the very poor absorption of codeine 6-glucuronide from the subcutaneous tissue.

After intravenous administration of codeine, some codeine was converted into morphine. Morphine 3-glucuronide was also detected in the plasma, but not morphine 6-glucuronide. There was also a small amount of codeine 6-glucuronide detected in the plasma. In the brain, both morphine 6-glucuronide and codeine 6-glucuronide were present. Codeine 6-glucuronide administration resulted in the presence of only codeine 6-glucuronide in the plasma, showing that it is stable in the plasma and does not convert to either codeine, morphine or morphine 6-glucuronide. In the brain, some codeine 6-glucuronide was seen to be O-demethylated to morphine 6-glucuronide. Therefore, the analgesic responses observed in the rats would appear to be associated with codeine 6-glucuronide and to some extent, morphine 6-glucuronide.

Codeine, although a chemical congener of morphine, is more widely used as an analgesic since it has substantially less potential for the development of dependence. It has been suggested that the analgesic action of codeine is associated with its conversion to morphine in the body. If this was the case however, one would expect to see relatively high concentrations of morphine in

the plasma and urine after codeine administration. However, only small amounts of morphine were detected after oral administration of codeine by various investigators (Quiding et al., 1986 ; Shah and Mason, 1990 a ; Persson et al., 1992). In addition, urinary excretion of morphine has been reported to be only 0.2-0.8% of the total codeine dose (Yue et al, 1990 a, b ; Chen et al., 1991). This is consistent with the report of Persson et al. (1992) that the ratio of the AUC of morphine to codeine is only 3%. Sindrup et al. (1990) found morphine concentrations of up to 13 ng/ml in extensive metabolizers 90 minutes after oral administration of 75 mg of codeine. In contrast, Findlay et al. (1986) and Guay et al. (1987) have reported concentrations of morphine up to 16 ng/ml after an oral dose of 60 mg of codeine, which theoretically could contribute to the analgesic effect of codeine.

Although these reports regarding morphine levels after codeine treatment are contradictory, it is important to recognize that high morphine concentrations were all obtained using radioimmunoassays for morphine (Findlay et al., 1986 ; Guay et al., 1987), while other studies in which low or undetectable concentrations were reported used either HPLC (Chen et al., 1991 ; Shah and Mason, 1991 ; Yue et al., 1990 a, b) or GC-MS (Quiding et al., 1986) techniques. The apparent high concentrations of morphine may therefore be associated with cross-reactivity of the antibody to other opiates. Thus, in view of the low morphine concentrations it is most likely that its contribution to the analgesic effect of codeine is small. However, it has been hypothesized that O-demethylation to morphine may take place in the vicinity of receptors in the CNS (Chen et al., 1990). It has also been suggested that codeine may act on different

opioid receptors (Neil, 1984) and, therefore, may itself mediate a large part of its analgesic effect.

There has been no documented study regarding the activity of codeine 6-glucuronide, the major metabolite in some species including humans. In contrast to the low concentrations of morphine produced after codeine administration in humans, concentrations of codeine 6-glucuronide have been reported to be 10-15 times those of codeine itself. We have shown that not only does codeine 6-glucuronide cross the blood brain barrier, it also has potent analgesic activity. This is in agreement with previous work describing the activity of morphine 6-glucuronide.

Yoshimura et al. (1973) reported that both morphine 6-glucuronide and morphine 3-glucuronide could cross the blood brain barrier and act on the opioid receptors in the brain of rats. Other animal studies done in both rat and mouse have shown that morphine 6-glucuronide has a higher affinity for μ opioid receptors than morphine itself (Abbott and Palmour, 1988 ; Pasternak et al., 1980 ; Paul et al., 1989 ; Gong et al., 1991) and is 2-4 times more potent as an analgesic than morphine when injected subcutaneously as assessed by antinociceptive tests, that is, tail flick and hot plate tests (Gong et al., 1992 ; Hanna et al., 1990).

Morphine 3-glucuronide, on the other hand, was found to have a very low affinity for μ opioid receptors in the brain and did not exhibit any analgesic effect when administered in pain-induced rat and mouse. Direct clinical evidence for the analgesic activity of morphine 6-glucuronide has been reported in man (Osborne et al., 1988 , 1990). It was seen that lower doses of morphine 6-glucuronide were needed compared to morphine for the same extent of pain

relief in cancer patients (Hanna et al., 1990 ; 1991). Our results show that like morphine 6-glucuronide, codeine 6-glucuronide possesses analgesic activity of its own.

The concept of opiates as immunomodulatory agents has long been recognized. It is well known that cell-mediated immune function is impaired in heroin and opiate addicts (Brown et al., 1974) and that the major cell types involved in cell-mediated immunity, that is, T lymphocytes and mononuclear phagocytes, possess opiate receptors (Wybran et al., 1979 ; McDonough et al., 1980 ; Lopker et al., 1980). Opiate addicts have been shown to exhibit an increased incidence of bacterial, protozoal, fungal and viral infections (Bryant et al., 1990). Until recently, this was attributed to the sharing of unsterilized and contaminated needles by the addicts. However, there is now a considerable body of evidence suggesting that opiates themselves cause suppression of various immunological endpoints.

The immunosuppressive effects of opiates may have potentially important clinical relevance, particularly in the case of individuals having a high degree of susceptibility towards infectious diseases. Effects on immunocompetence is a major area of concern when dealing with organ transplants, burns, cancer, rheumatoid arthritis and other autoimmune diseases.

The relationship between the receptor binding affinity of codeine 6-glucuronide and its *in vivo* potency seems to be inconsistent. The glucuronide is analgesically much more active than its parent compound, but its receptor affinity does not reflect this observation. There are a few possible explanations. First, binding studies performed with brain homogenates do not necessarily represent the true physiological conditions. At best they can give a good approximation

regarding the activity of the compounds. They only indicate the binding of compounds to receptors under the specified conditions of the assay. However, this does not accurately estimate the efficacy of the compounds *in vivo* in producing a physiological response. Second, differences in the distribution, metabolism and elimination of these compounds can play a major role in modulating the pharmacodynamic responses. This too cannot be taken into account in binding studies. Third, these compounds may possess higher affinity towards other opioid (delta, kappa etc.) or even non-opioid receptors and this interaction may account for the enhanced analgesic potency of codeine 6-glucuronide *in vivo*. The lesser affinity of the glucuronide for the μ receptor may also account for its lower immunosuppressive effects.

Shashoua et al. (1986) have identified UDP-glucuronic acid, a co-substrate for the glucuronidation reaction, in the rodent brain. There is also evidence of the presence of glucuronosyl transferase enzymes in the brain of rodents and humans (Wahlstrom et al., 1988). This raises the possibility that glucuronidation occurs in the brain. At present this seems unlikely and further investigation needs to be done in order to validate this hypothesis. It is more reasonable to assume that codeine 6-glucuronide produced in the liver is able to cross the blood brain barrier by an, as yet, unknown mechanism.

The unexpected behavior of the hydrophilic conjugates of codeine and morphine with regards to crossing the blood brain barrier was partly explained by Carrupt et al. (1991). It was suggested that glucuronides existed in a conformational equilibrium between their hydrophilic and lipophilic forms, depending on the surrounding media. In the vicinity of the BBB the glucuronides were seen to be present in their lipophilic form which allowed easier penetration

into the brain. As the glucuronides circulating in the blood were in much higher concentration compared to their parent compounds, this caused a sufficient concentration gradient to be established, so as to allow the glucuronides to passively diffuse across the blood brain barrier (Barjavel et al., 1995).

In conclusion, the data presented in this thesis suggests that the glucuronide metabolites of codeine and morphine may be more beneficial in the treatment of patients with low immunocompetence experiencing clinical pain during trauma and even after major surgery. Hence, both codeine 6-glucuronide and morphine 6-glucuronide, along with related compounds like the intermediate, deserve further assessment as potential agents for clinical pain management in the case of patients with a compromised immune function.

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BIOGRAPHICAL SKETCH

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